Use of astaxanthin-containing plants or parts of plants of the genus Tagetes as feedstuffs

Description

The present invention relates to the use of astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes for oral administration to animals, methods for producing animal feed preparations, the animal feed preparations themselves, a method for pigmenting animals or animal products, and also a method for producing pigmented animals and animal products.

On account of its coloring properties astaxanthin is used as pigment in animal nutrition, in particular in trout, salmon and shrimp breeding.

Astaxanthin is currently chiefly produced by chemical synthesis methods. Natural astaxanthin is currently produced in biotechnological methods in small amounts by culturing algae, for example *Haematococcus pluvialis*, or by fermentation of genetically optimized microorganisms and subsequent isolation.

Synthetic astaxanthin or natural astaxanthin produced by isolation is chemically and/or physically stabilized by special formulation techniques for increasing storage life and is prepared for the respective use in accordance with the desired application sectors and bioavailabilities.

WO 9201754 describes an astaxanthin-containing wild type plant of the species Adonis aestivalis. In addition, the document discloses the use of the astaxanthin-containing petals of Adonis aestivalis and also extracts thereof as fish food, or as additive in fish food for pigmenting fish.

The use of Adonis aestivalis as a plant source of astaxanthin for pigmenting fish in the prior art, however, has the disadvantage that the yield of astaxanthin-containing biomass and thus of astaxanthin-containing plant material per unit of culture area is very low, and thus a satisfactory amount of astaxanthin-containing plant material can only be obtained by cost-intensive culture of large areas. This leads to high costs in the production of corresponding pigments.

It was therefore an object of the invention to provide pigmenting agents which no longer have the disadvantage of the prior art.

Accordingly it has been found that astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes can be used for oral administration to animals.

In a preferred embodiment, the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes are used for pigmenting animals and the corresponding animal products.

Astaxanthin-containing plants of the genus Tagetes are preferably taken to mean plants of the genus Tagetes which have a content of astaxanthin in at least one part of the plant. The astaxanthin can be present in free form in the form of fatty acid diesters or monoesters. Preferred plants of the genus Tagetes are plants selected from the species Tagetes erecta, Tagetes patula, which are also termed Marigold, Tagetes lucida, Tagetes pringlei, Tagetes palmeri, Tagetes minuta, Tagetes lemmonii, Tagetes tenuifolia, or Tagetes campanulata, particularly preferably *Tagetes erecta* or *Tagetes patula*.

Astaxanthin-containing parts of plants of plants of the genus Tagetes are preferably taken to mean parts of plants which have a content of astaxanthin in at least one part of the plant part. Preferred plant parts are, for example, flowers, flower heads or, particularly preferably, flower leaves which are also called petals.

Wild type plants of the genus Tagetes do not have astaxanthin in flowers, but do have carotenoids such as lutein and zeaxanthin. However, it has been found according to the invention that the plants of the genus Tagetes can be given the capacity to produce astaxanthin, for example by genetic modification.

In a preferred embodiment, the plants of the genus Tagetes are given the capacity to produce astaxanthin, for example, by causing a ketolase activity in the plants of the genus Tagetes which have been genetically modified compared with the wild type.

Ketolase activity is taken to mean the enzyme activity of a ketolase.

A ketolase is taken to mean a protein which has the enzymatic activity to introduce a keto group at the optionally substituted β-ionone ring of carotenoids.

In particular, a ketolase is taken to mean a protein which has the enzymatic activity to convert β-carotene into canthaxanthin.

Accordingly, ketolase activity is taken to mean the amount of β-carotene converted or amount of canthaxanthin formed in a defined time by the protein ketolase.

The term "wild type" is taken to mean according to the invention the corresponding non-genetically modified starting plant of the genus Tagetes.

Depending on context, the term "plant" can be taken to mean the starting plant (wild type) of the genus Tagetes or an inventive genetically modified plant of the genus Tagetes, or both.

Preferably, "wild type" is taken to mean a reference plant in each case for the production of the ketolase activity, for the hereinafter described increase of the hydroxylase activity, for the hereinafter described increase of the β-cyclase activity, and for the hereinafter described reduction of the ε-cyclase activity and the increase of the astaxanthin content.

This reference plant of the genus Tagetes is Tagetes erecta, Tagetes patula, Tagetes lucida, Tagetes pringlei, Tagetes palmeri, Tagetes minuta or Tagetes campanulata, particularly preferably *Tagetes erecta,* very particularly preferably Tagetes erecta L., Accession number: TAG 72, cultivar Orangenprinz, available from the IPK Genebank, Corrensstr. 3, D-06466 Gatersleben.

The ketolase activity in inventive genetically modified plants of the genus Tagetes and in wild type or reference plants is preferably determined under the following conditions:

The ketolase activity in plant material is determined on the basis of the method of Frazer et al., (J. Biol. Chem. 272(10): 6128-6135, 1997). The ketolase activity in plant extracts is determined using the substrates beta-carotene and canthaxanthin in the

presence of lipid (soylecithin) and detergent (sodium cholate). Substrate/product ratios from the ketolase assays are determined by means of HPLC.

The inventive genetically modified plant of the genus Tagetes has, in this preferred embodiment, compared to the nongenetically modified wild type, a ketolase activity, preferably in flower leaves, and is thus preferably able to express a ketolase transgenically.

In a further preferred embodiment, the ketolase activity in the plants of the genus Tagetes is brought about by bringing about the gene expression of a nucleic acid coding for a ketolase.

In this preferred embodiment, the gene expression of a nucleic acid coding for a ketolase is preferably brought about by introducing nucleic acids which code for ketolases in the starting plant of the genus Tagetes.

For this, in principle, any ketolase gene, that is to say any nucleic acids which code for a ketolase, can be used.

All nucleic acids mentioned in the description can be, for example, an RNA, DNA or cDNA sequence.

With genomic ketolase sequences from eukaryotic sources which comprise introns, in the event that the host plant of the genus Tagetes is not able to, or cannot be given the ability to, express the corresponding ketolase, preferably already-processed nucleic acid sequences such as the corresponding cDNAs are to be used.

Examples of nucleic acids coding for a ketolase and the corresponding ketolases which can be used in the inventive method are, for example, sequences from

Haematoccus pluvialis, in particular from Haematoccus pluvialis Flotow em. Wille (Accession NO: X86782; nucleic acid: SEQ ID NO: 1, protein SEQ ID NO: 2),

Haematoccus pluvialis, NIES-144 (Accession NO: D45881; nucleic acid: SEQ ID NO: 3, protein SEQ ID NO: 4),

Agrobacterium aurantiacum (Accession NO: D58420; nucleic acid: SEQ ID NO: 5, protein SEQ ID NO: 6),

Alicaligenes spec. (Accession NO: D58422; nucleic acid: SEQ ID NO: 7, protein SEQ ID NO: 8),

Paracoccus marcusii (Accession NO: Y15112; nucleic acid: SEQ ID NO: 9, protein SEQ ID NO: 10).

Synechocystis sp. Strain PC6803 (Accession NO: NP442491; nucleic acid: SEQ ID NO: 11, protein SEQ ID NO: 12).

Bradyrhizobium sp. (Accession NO: AF218415; nucleic acid: SEQ ID NO: 13, protein SEQ ID NO: 14).

Nostoc sp. Strain PCC7120 (Accession NO: AP003592, BAB74888; nucleic acid: SEQ ID NO: 15, protein SEQ ID NO: 16),

Nostoc punctiforme ATTC 29133, nucleic acid: Acc. No. NZ_AABC01000195, base pair 55,604 to 55,392 (SEQ ID NO: 81); protein: Acc. No. ZP_00111258 (SEQ ID NO: 82) (annotated as putative protein),

Nostoc punctiforme ATTC 29133, nucleic acid: Acc. No. NZ_AABC01000196, base pair 140,571 to 139,810 (SEQ ID NO: 83), protein: (SEQ ID NO: 84) (not annotated),

Synechococcus sp. WH 8102, nucleic acid: Acc. No. NZ_AABD01000001, base pair 1,354,725-1,355,528 (SEQ ID NO: 85), protein: Acc. No. ZP_00115639 (SEQ ID NO: 86) (annotated as putative protein),

Haematococcus pluvialis (Accession NO: AF534876, AAN03484; nucleic acid: SEQ ID NO: 97, protein: SEQ ID NO: 98),

Paracoccus sp. MBIC1143, (Accession NO: D58420, P54972; nucleic acid: SEQ ID NO: 99, protein: SEQ ID NO: 100),

Brevundimonas aurantiaca (Accession NO: AY166610, AAN86030; nucleic acid: SEQ ID NO: 101, protein: SEQ ID NO: 102),

Nodularia spumigena NSOR10 (Accession NO: AY210783, AAO64399; nucleic acid: SEQ ID NO: 103, protein: SEQ ID NO: 104) and

Deinococcus radiodurans R1 (Accession NO: E75561, AE001872; nucleic acid: SEQ ID NO: 105, protein: SEQ ID NO: 106).

Further natural examples of ketolases and ketolase genes which can be used in the inventive method may be readily found, for example, from various organisms whose genomic sequence is known by comparing the identity of amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the above-described sequences and, in particular, with the sequences SEQ ID NO: 2 and/or 16.

Further natural examples of ketolases and ketolase genes may in addition be readily found starting from the above-described nucleic acid sequences, in particular starting from the sequences SEQ ID NO: 2 and/or 16 from various organisms whose genomic sequence is not known by hybridization techniques in a manner known per se.

The hybridization can be performed under moderate (low stringency), or preferably under stringent (high stringency) conditions.

Such hybridization conditions are described, for example in Sambrook, J., Fritsch, E.F., Maniatis, T., in: Molecular Cloning (A Laboratory Manual), 2nd edition, Cold Spring Harbor Laboratory Press, 1989, pages 9.31-9.57 or in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6.

For example, the conditions during the washing step can be selected from the range of conditions limited by those of low stringency (with 2 × SSC at 50°C) and those of high stringency (with 0.2 × SSC at 50°C, preferably at 65°C) (20 × SSC: 0.3 M sodium citrate, 3 M sodium chloride, pH 7.0).

Furthermore, the temperature during the washing step can be elevated from moderate conditions at room temperature, 22°C, to stringent conditions at 65°C.

Both parameters, salt concentration and temperature, can be varied simultaneously, and also one of the two parameters can be kept constant and only the other varied. During the hybridization, denaturing agents, for example formamide or SDS, can also be used. In the presence of 50% formamide, the hybridization is preferably carried out at 42°C.

Some exemplary conditions for hybridization and washing step are given as follows:

(1) hybridization conditions having, for example

(i) 4 × SSC at 65°C, or

(ii) 6 × SSC at 45°C, or

(iii) 6 × SSC at 68°C, 100 mg/ml of denatured fish sperm DNA, or

(iv) 6 × SSC, 0.5% SDS, 100 mg/ml of denatured fragmented salmon sperm DNA at 68°C, or

(v) 6 × SSC, 0.5% SDS, 100 mg/ml of denatured fragmented salmon sperm DNA, 50% formamide at 42°C, or

(vi) 50% formamide, 4 × SSC at 42°C, or

(vii) 50% (vol/vol) formamide, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer pH 6.5, 750 mM NaCl, 75 mM sodium citrate at 42°C, or

(viii) 2 × or 4 × SSC at 50°C (moderate conditions), or

(ix) 30 to 40% formamide, 2 × or 4 × SSC at 42° (moderate conditions).

(2) Wash steps for 10 minutes in each case, using for example

(i) 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C, or

(ii) 0.1 × SSC at 65°C, or

(iii) 0.1 × SSC, 0.5% SDS at 68°C, or

(iv) 0.1 × SSC, 0.5% SDS, 50% formamide at 42°C, or

(v) 0.2 × SSC, 0.1% SDS at 42°C, or

(vi) 2 ×SSC at 65°C (moderate conditions).

In a preferred embodiment of the inventive genetically modified plants of the genus Tagetes, nucleic acids are introduced, which nucleic acids code for a protein comprising the amino acid sequence SEQ ID NO: 2 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which sequence has an identity of at least 20%, preferably at least 30%, more preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, particularly preferably at least 90%, at the amino acid level with the sequence SEQ ID NO: 2 and has the enzymatic property of a ketolase.

A natural ketolase sequence can be involved which, as described above, can be found by comparison of identity of the sequences from other organisms, or a synthetic ketolase sequence which has been modified, starting from the sequence SEQ ID NO: 2 by artificial variation, for example by substitution, insertion or deletion of amino acids.

In a further preferred embodiment of the inventive method, nucleic acids are introduced which code for a protein comprising the amino acid sequence SEQ ID NO: 16 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which sequence has an identity of at least 20%, preferably at least 30%, more preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, particularly preferably at least 90%, at the amino acid level, with the sequence SEQ ID NO: 16 and the enzymatic property of a ketolase.

A natural ketolase sequence can be involved which, as described above, can be found by comparison of identity of the sequences from other organisms, or a synthetic

ketolase sequence which has been modified starting from the sequence SEQ ID NO: 16 by artificial variation, for example by substitution, insertion or deletion of amino acids.

The term "substitution" is to be taken to mean in the description the exchange of one or more amino acids by one or more amino acids. Preferably, what are termed conservative exchanges are carried out, in which the amino acid replaced has a similar property to the original amino acid, for example exchange of Glu for Asp, Gln for Asn, Val for Ile, Leu for Ile, Ser for Thr.

Deletion is the replacement of an amino acid by a direct bond. Preferred positions for deletions are the termini of the polypeptide and the linkages between the individual protein domains.

Insertions are introductions of amino acids into the polypeptide chain, formally, a direct bond being replaced by one or more amino acids.

Identity between two proteins is taken to mean the identity of the amino acids over the whole protein length in each case, in particular the identity which is calculated by comparison using the Lasergene Software from DNASTAR, inc. Madison, Wisconsin (USA) using the Clustal method (Higgins DG, Sharp PM. Fast and sensitive multiple sequence alignments on a microcomputer. Comput Appl. Biosci. 1989 Apr;5(2):151-1) with the following parameters being set:

Multiple alignment parameter:

Gap penalty	10
Gap length penalty	10

Pairwise alignment parameter:

K-tuple	1
Gap penalty	3
Window	5
Diagonals saved	5

A protein which has an identity of at least 20% at the amino acid level with the sequence SEQ ID NO: 2 or 16 is correspondingly taken to mean a protein which, in a comparison of its sequence with the sequence SEQ ID NO: 2 or 16, in particular

according to the above program logarithm with the above parameter set, has an identity of at least 20%.

Suitable nucleic acid sequences are, for example, obtainable by back-translation of the polypeptide sequence according to the genetic code.

Preferably, for this, those codons are used which are used frequently in accordance with the tagetes-specific codon usage. The codon usage may be readily determined on the basis of computer evaluations of other known genes from plants of the genus Tagetes.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 1 is introduced into the plant of the genus.

In a further, particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 15 is introduced into the plant of the genus.

All of the abovementioned ketolase genes can be produced, furthermore, in a manner known per se by chemical synthesis from the nucleotide building blocks, for example by fragment condensation of individual overlapping complementary nucleic acid building blocks of the double helix. The chemical synthesis of oligonucleotides can be performed, for example, in a known manner by the phosphoamidite method (Voet, Voet, 2nd edition, Wiley Press New York, pp. 896-897). The addition of synthetic oligonucleotides and filling-in of gaps using the Klenow fragment of DNA polymerase and ligation reactions and also general cloning methods are described in Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press.

In a particularly preferred embodiment of the inventive method, use is made of genetically modified plants of the genus Tagetes which, in flowers, have the highest expression rate of a ketolase.

Preferably, this is achieved by the ketolase gene being expressed under control of a flower-specific promoter. For example, for this, the above-described nucleic acids, as described extensively hereinafter, in a nucleic acid construct, functionally linked with a flower-specific promoter, are introduced into the plant of the genus Tagetes.

Particularly preferred plants of the genus Tagetes as starting plants, or inventive genetically modified plants, are plants selected from the species Tagetes erecta, Tagetes patula, which are also termed Marigold, Tagetes lucida, Tagetes pringlei, Tagetes palmeri, Tagetes minuta, Tagetes lemmonii, Tagetes tenuifolia, or Tagetes campanulata, particularly preferably *Tagetes erecta* or *Tagetes patula.*

In a preferred embodiment, use is made of genetically modified plants of the genus Tagetes which, compared with the wild type, additionally have an elevated hydroxylase activity and/or β-cyclase activity.

Hydroxylase activity is taken to mean the enzyme activity of a hydroxylase.

A hydroxylase is taken to mean a protein which has the enzymatic activity to introduce a hydroxyl group on the optionally substituted β-ionone ring of carotenoids.

In particular, a hydroxylase is taken to mean a protein which has the enzymatic activity to convert, β-carotene to zeaxanthin, or canthaxanthin to astaxanthin.

Accordingly, hydroxylase activity is taken to mean the amount of β-carotene or canthaxanthin converted, or amount of zeaxanthin or astaxanthin formed, in a defined time by the protein hydroxylase.

At an elevated hydroxylase activity compared with the wild type, thus, compared with the wild type, the amount of β-carotene or canthaxanthin converted, or the amount of zeaxanthin or astaxanthin formed by the protein hydroxylase in a defined time is increased.

Preferably, this increase of hydroxylase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the hydroxylase activity of the wild type.

β-Cyclase activity is taken to mean the enzyme activity of a β-cyclase.

β-Cyclase is taken to mean a protein which has the enzymatic activity to convert a terminal linear residue of lycopene to a β-ionone ring.

In particular, β-cyclase is taken to mean a protein which has the enzymatic activity to convert γ-carotene to β-carotene.

Accordingly, β-cyclase activity is taken to mean the amount of β-carotene converted or amount of β-carotene formed by the protein β-cyclase in a defined time.

At an elevated β-cyclase activity compared with the wild type, thus the amount of γ-carotene converted, or the amount of β-carotene formed, is increased by the protein β-cyclase in a defined time compared with the wild type.

Preferably, this increase of β-cyclase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the β-cyclase activity of the wild type.

The hydroxylase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

The activity of the hydroxylase is determined *in vitro* in accordance with Bouvier et al. (Biochim. Biophys. Acta 1391 (1998), 320-328). Ferredoxin, ferredoxin-NADP oxidoreductase, catalase, NADPH and beta-carotene together with mono- and digalactosyl glycerides are added to a defined amount of plant extract.

Particularly preferably, the hydroxylase activity is determined under the following conditions according to Bouvier, Keller, d'Harlingue and Camara (Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (Capsicum annuum L.; Biochim. Biophys. Acta 1391 (1998), 320-328):

The in vitro assay is carried out in a volume of 0.250 ml. The assay mix comprises 50 mM potassium phosphate (pH 7.6), 0.025 mg of spinach ferredoxin, 0.5 units of ferredoxin-$NADP^+$ spinach oxidoreductase, 0.25 mM NADPH, 0.010 mg of beta-

carotene (emulsified in 0.1 mg of Tween 80), 0.05 mM of a mixture of mono- and digalactosyl glycerides (1:1), 1 unit of catalyse, 200 mono- and digalactosyl glycerides, (1:1), 0.2 mg of bovine serum albumin and plant extract in differing volumes. The reaction mixture is incubated at 30°C for 2 hours. The reaction products are extracted with organic solvent such as acetone or chloroform/methanol (2:1) and determined by means of HPLC.

The β–cyclase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

The β-cyclase activity is determined *in vitro* in accordance with Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15). To a defined amount of plant extract are added potassium phosphate buffer (pH 7.6), lycopene as substrate, paprika stroma protein, $NADP^+$, NADPH and ATP.

Particularly preferably, the hydroxylase activity is determined under the following conditions according to Bouvier, d'Harlingue and Camara (Molecular Analysis of carotenoid cyclae inhibition; Arch. Biochem. Biophys. 346(1) (1997) 53-64):

The in vitro assay is carried out in a volume of 250 µl. The assay mix comprises 50 mM potassium phosphate (pH 7.6), differing amounts of plant extract, 20 nM lycopene, 250 µg of paprika chromoplastid stroma protein, 0.2 mM $NADP^+$, 0.2 mM NADPH and 1 mM ATP. NADP/NADPH and ATP are dissolved in 10 ml of ethanol together with 1 mg of Tween 80 immediately before addition to incubation medium. After a reaction time of 60 minutes at 30°C, the reaction is ended by adding chloroform/methanol (2:1). The reaction products extracted in chloroform are analyzed by means of HPLC.

An alternative assay using radioactive substrate is described in Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15).

The hydroxylase activity and/or β-cyclase activity can be increased by various ways, for example by switching off inhibiting regulatory mechanisms at the expression and protein level, or by increasing the gene expression, compared with the wild type, of nucleic acids coding for a hydroxylase and/or nucleic acids coding for a β-cyclase.

The gene expression of the nucleic acids coding for a hydroxylase and/or the gene expression of the nucleic acid coding for a β-cyclase can likewise be increased compared with the wild type by various ways, for example by inducing the hydroxylase gene and/or β-cyclase gene by activators or by introducing one or more hydroxylase gene copies and/or β-cyclase gene copies, that is to say by introducing at least one nucleic acid coding for a hydroxylase and/or at least one nucleic acid coding for an ε-cyclase into the plant of the genus Tagetes.

Increasing the gene expression of a nucleic acid coding for a hydroxylase and/or β-cyclase is also taken to mean, according to the invention, the manipulation of the expression of the endogenous hydroxylase and/or β-cyclase of the plants of the genus Tagetes.

This can be achieved, for example, by modifying the genes coding for promoter DNA sequence for hydroxylases and/or β-cyclases. Such a modification which causes an increased expression rate of the gene, can be performed, for example, by deletion or insertion of DNA sequences.

It is possible, as described above, to change the expression of the endogenous hydroxylase and/or β-cyclase by applying exogenous stimuli. This can be performed by particular physiological conditions, that is to say by applying foreign substances.

Furthermore, an altered or increased expression of an endogenous hydroxylase and/or β-cyclase gene can be achieved by a regulator protein which does not occur in the non-transformed plant interacting with the promoter of this gene.

Such a regulator can be a chimeric protein which consists of a DNA-binding domain and a transcription activator domain, as described, for example, in WO 96/06166.

In a preferred embodiment, the gene expression of a nucleic acid coding for a hydroxylase and/or the gene expression of a nucleic acid coding for a β-cyclase is increased by introducing at least one nucleic acid coding for a hydroxylase and/or by introducing at least one nucleic acid coding for a β-cyclase into the plant of the genus Tagetes.

For this, in principle, use can be made of any hydroxylase gene or any β-cyclase gene, that is to say any nucleic acid which codes for a hydroxylase and any nucleic acid which codes for a β-cyclase.

With genomic hydroxylase or β-cyclase nucleic acid sequences from eukaryotic sources which comprise introns, in the event that the host plant does not have the capacity to, or cannot be given the capacity to, express the corresponding hydroxylase or β-cyclase, previously-processed nucleic acid sequences, such as the corresponding cDNAs are preferably to be used.

An example of a hydroxylase gene is a nucleic acid coding for a hydroxylase from Haematococcus pluvialis (Accession AX038729, WO 0061764); (nucleic acid: SEQ ID NO: 17, protein: SEQ ID NO: 18).

and also hydroxylases of the following accession numbers:

|emb|CAB55626.1, CAA70427.1, CAA70888.1, CAB55625.1, AF499108_1, AF315289_1, AF296158_1, AAC49443.1, NP_194300.1, NP_200070.1, AAG10430.1, CAC06712.1, AAM88619.1, CAC95130.1, AAL80006.1, AF162276_1, AAO53295.1, AAN85601.1, CRTZ_ERWHE, CRTZ_PANAN, BAB79605.1, CRTZ_ALCSP, CRTZ_AGRAU, CAB56060.1, ZP_00094836.1, AAC44852.1, BAC77670.1, NP_745389.1, NP_344225.1, NP_849490.1, ZP_00087019.1, NP_503072.1, NP_852012.1, NP_115929.1, ZP_00013255.1

A particularly preferred hydroxylase is in addition the hydroxylase from tomato (Accession Y14809) (nucleic acid: SEQ ID NO: 107; protein: SEQ ID NO. 108).

Examples of β-cyclase genes are:
a nucleic acid coding for a β-cyclase from tomato (Accession X86452) (nucleic acid: SEQ ID NO: 19, protein: SEQ ID NO: 20).

And also β-cyclases of the following accession numbers:

S66350 lycopene beta-cyclase (EC 5.5.1.-) - tomato
CAA60119 lycopene synthase [Capsicum annuum]

S66349 lycopene beta-cyclase (EC 5.5.1.-) - common tobacco

CAA57386 lycopene cyclase [Nicotiana tabacum]

AAM21152 lycopene beta-cyclase [Citrus sinensis]

AAD38049 lycopene cyclase [Citrus x paradisi]

AAN86060 lycopene cyclase [Citrus unshiu]

AAF44700 lycopene beta-cyclase [Citrus sinensis]

AAK07430 lycopene beta-cyclase [Adonis palaestina]

AAG10429 beta-cyclase [Tagetes erecta]

AAA81880 lycopene cyclase

AAB53337 lycopene beta-cyclase

AAL92175 beta-lycopene cyclase [Sandersonia aurantiaca]

CAA67331 lycopene cyclase [Narcissus pseudonarcissus]

AAM45381 beta-cyclase [Tagetes erecta]

AAO18661 lycopene beta-cyclase [Zea mays]

AAG21133 chromoplast-specific lycopene beta-cyclase [Lycopersicon esculentum]

AAF18989 lycopene beta-cyclase [Daucus carota]

ZP_001140 hypothetical protein [Prochlorococcus marinus str. MIT9313]

ZP_001050 hypothetical protein [Prochlorococcus marinus subsp. pastoris str. CCMP1378]

ZP_001046 hypothetical protein [Prochlorococcus marinus subsp. pastoris str. CCMP1378]

ZP_001134 hypothetical protein [Prochlorococcus marinus str. MIT9313]

ZP_001150 hypothetical protein [Synechococcus sp. WH 8102]

AAF10377 lycopene cyclase [Deinococcus radiodurans]

BAA29250 393aa long hypothetical protein [Pyrococcus horikoshii]

BAC77673 lycopene beta-monocyclase [marine bacterium P99-3]

AAL01999 lycopene cyclase [Xanthobacter sp. Py2]

ZP_000190 hypothetical protein [Chloroflexus aurantiacus]

ZP_000941 hypothetical protein [Novosphingobium aromaticivorans]

AAF78200 lycopene cyclase [Bradyrhizobium sp. ORS278]

BAB79602 crtY [Pantoea agglomerans pv. milletiae]

CAA64855 lycopene cyclase [Streptomyces griseus]

AAA21262 lycopene cyclase [Pantoea agglomerans]

C37802 crtY protein - Erwinia uredovora

BAB79602 crtY [Pantoea agglomerans pv. milletiae]

AAA64980 lycopene cyclase [Pantoea agglomerans]
AAC44851 lycopene cyclase
BAA09593 lycopene cyclase [Paracoccus sp. MBIC1143]
ZP_000941 hypothetical protein [Novosphingobium aromaticivorans]
CAB56061 lycopene beta-cyclase [Paracoccus marcusii]
BAA20275 lycopene cyclase [Erythrobacter longus]
ZP_000570 hypothetical protein [Thermobifida fusca]
ZP_000190 hypothetical protein [Chloroflexus aurantiacus]
AAK07430 lycopene beta-cyclase [Adonis palaestina]
CAA67331 lycopene cyclase [Narcissus pseudonarcissus]
AAB53337 lycopene beta-cyclase
BAC77673 lycopene beta-monocyclase [marine bacterium P99-3]

A particularly preferred β-cyclase is, in addition, the chromoplast-specific β-cyclase from tomato (AAG21133) (nucleic acid: SEQ ID No. 109; protein: SEQ ID No. 110).

In the inventive preferred transgenic plants of the genus Tagetes, there is therefore, in this preferred embodiment, compared with the wild type, at least one further hydroxylase gene and/or β-cyclase gene.

In this preferred embodiment the genetically modified plant has, for example, at least one exogenous nucleic acid coding for a hydroxylase, or at least two endogenous nucleic acids coding for a hydroxylase, and/or at least one exogenous nucleic acid coding for a β-cyclase, or at least two endogenous nucleic acids coding for a β-cyclase.

Preferably, in the above-described preferred embodiment, use is made of, as hydroxylase genes, nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 18 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 18, and which have the enzymatic property of a hydroxylase.

Further examples of hydroxylases and hydroxylase genes may be readily found, for example, from various organisms whose genomic sequence is known, as described

above, by comparisons of homology of the amino acid sequences or the corresponding back-translated nucleic acid sequences from databases with the SEQ ID. NO: 18.

Further examples of hydroxylases and hydroxylase genes may further be readily found, for example, starting from the sequence SEQ ID NO: 17 from various organisms whose genomic sequence is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the hydroxylase activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the hydroxylase of the sequence SEQ ID NO: 18.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence according to the genetic code.

Preferably, for this, those codons are used which are frequently used in accordance with the plant-specific codon usage. The codon usage may be determined readily on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 17 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as β-cyclase genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 20 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level with the sequence SEQ ID NO: 20 and which have the enzymatic property of a β-cyclase.

Further examples of β-cyclases and β-cyclase genes may readily be found, for example, from various organisms whose genomic sequence is known, as described above, by comparisons of homology of the amino acid sequences or the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 20.

Further examples of β-cyclases and β-cyclase genes may further readily be found, for example, starting from the sequence SEQ ID NO: 19 from various organisms whose genomic sequence is not known, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the β-cyclase activity, nucleic acids are introduced into organisms which code for proteins comprising the amino acid sequence of the β-cyclase of the sequence SEQ ID NO: 20.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence according to the genetic code.

Preferably, for this, those codons are used which are used frequently in accordance with the plant-specific codon usage. The codon usage may be determined readily on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 19 is introduced into the organism.

All abovementioned hydroxylase genes or β-cyclase genes can be produced, furthermore, in a manner known per se by chemical synthesis from the nucleotide building blocks, for example by fragment condensation of individual overlapping complementary nucleic acid building blocks of the double helix. The chemical synthesis of oligonucleotides can be performed, for example, in a known manner by the phosphoamidite method (Voet, Voet, 2nd edition, Wiley Press New York, pages 896-897). The addition of synthetic oligonucleotides and filling-in of gaps using the Klenow fragment of DNA polymerase and ligation reactions and general cloning methods are described in Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press.

In a further preferred embodiment of the method, the plants of the genus Tagetes have, compared to the wild type, in addition, a reduced ε-cyclase activity.

ε-Cyclase activity is taken to mean the enzyme activity of an ε-cyclase.

An ε-cyclase is taken to mean a protein which has the enzymatic activity to convert a terminal linear residue of lycopene into an ε-ionone ring.

An ε-cyclase is therefore taken to mean, in particular, a protein which has the enzymatic activity to convert lycopene to δ-carotene.

Accordingly, ε-cyclase activity is taken to mean the amount of lycopene converted or amount of δ-carotene formed by the protein ε-cyclase in a defined time.

With an ε-cyclase activity which is reduced compared with the wild type, thus, compared with the wild type, the amount of lycopene converted, or the amount of δ-carotene formed, is reduced by the protein ε-cyclase in a defined time.

A reduced ε-cyclase activity is preferably taken to mean the partial or essentially complete suppression or blocking, based on differing mechanisms of cell biology, of the functionality of an ε–cyclase in a plant cell, plant or part derived therefrom, tissue, organ, cells or seed.

The ε-cyclase activity in plants can be reduced compared with the wild type, for example, by reducing the amount of ε-cyclase protein, or the amount of ε-cyclase mRNA in the plant. Accordingly, an ε-cyclase activity which is reduced compared with the wild type can be determined directly, or via the determination of the amount of ε-cyclase protein or the amount of ε-cyclase mRNA of the inventive plant, in comparison with the wild type.

A reduction of the ε-cyclase activity comprises a quantitative decrease of an ε–cyclase up to an essentially complete absence of the ε–cyclase (that is to say lack of detectability of ε–cyclase activity or lack of immunological detectability of the ε–cyclase). Preferably, the ε–cyclase activity (or the amount of ε-cyclase protein or the amount of ε-cyclase mRNA) in the plant, particularly preferably in flowers, is reduced, in comparison with the wild type, by at least 5%, further preferably by at least 20%, further preferably by at least 50%, further preferably by 100%. In particular, "reduction" also means the complete absence of ε–cyclase activity (or of the ε-cyclase protein or of ε-cyclase mRNA).

The ε-cyclase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

The ε-cyclase activity can be determined *in vitro* in accordance with Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15), when, to a defined amount of plant extract, there are added potassium phosphate as buffer (pH 7.6), lycopene as substrate, paprika stroma protein, $NADP^+$, NADPH and ATP.

The ε-cyclase activity in inventive genetically modified plants and in wild type or reference plants is determined, particularly preferably, in accordance with Bouvier, d'Harlingue and Camara (Molecular Analysis of carotenoid cyclase inhibition; Arch. Biochem. Biophys. 346(1) (1997) 53-64):

The in vitro assay is carried out in a volume of 0.25 ml. The assay mix comprises 50 mM potassium phosphate (pH 7.6), differing amounts of plant extract, 20 nM lycopene, 0.25 mg of paprika chromoplastid stroma protein, 0.2 mM $NADP^+$, 0.2 mM NADPH and 1 mM ATP. NADP/NADPH and ATP are dissolved in 0.01 ml of ethanol together with 1 mg of Tween 80 immediately before addition to the incubation medium. After a reaction time of 60 minutes at 30°C, the reaction is terminated by addition of chloroform/methanol (2:1). The reaction products extracted in chloroform are analyzed by means of HPLC.

An alternative assay using radioactive substrate is described in Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15). A further analytical method is described in Beyer, Kröncke and Nievelstein (On the mechanism of the lycopene isomerase/cyclase reaction in Narcissus pseudonarcissus L. chromoplast; J. Biol. Chem. 266(26) (1991) 17072-17078).

Preferably the ε–cyclase activity in plants is reduced by at least one of the following methods:

a) introduction of at least one double-stranded ε–cyclase ribonucleic acid sequence, also termed hereinafter ε–cyclase-dsRNA, or an expression cassette ensuring expression thereof, or expression cassettes. Those methods in which the

ε–cyclase-dsRNA is directed toward an ε–cyclase gene (that is to say genomic DNA sequences such as the promoter sequence) or an ε–cyclase transcript (that is to say mRNA sequences) are comprised

b) introduction of at least one ε–cyclase antisense ribonucleic acid sequence, also termed hereinafter ε–cyclase-antisense RNA, or an expression cassette ensuring expression thereof. Those methods in which the ε–cyclase-antisense RNA is directed toward an ε–cyclase gene (that is to say genomic DNA sequences) or an ε–cyclase gene transcript (that is to say RNA sequences) are comprised. α–Anomeric nucleic acid sequences are also comprised

c) introduction of at least one ε–cyclase-antisense RNA combined with a ribozyme or an expression cassette ensuring expression thereof

d) introduction of at least one ε–cyclase sense ribonucleic acid sequence, also termed hereinafter ε–cyclase-sense RNA, for induction of a cosuppression, or of an expression cassette ensuring expression thereof

e) introduction of at least one DNA- or protein-binding factor against an ε–cyclase gene, RNA, or protein, or of an expression cassette ensuring expression thereof

f) introduction of at least one viral nucleic acid sequence causing ε–cyclase RNA breakdown, or of an expression cassette ensuring expression thereof

g) introduction of at least one construct for producing a loss of function, such as the generation of stop codons or a shift in the reading frame, on an ε–cyclase gene, for example, by producing an insertion, deletion, inversion or mutation in an ε–cyclase gene. Preferably, knockout mutants can be generated by means of targeted insertion into said ε–cyclase gene by homologous recombination or introduction of sequence-specific nucleases against ε–cyclase gene sequences.

It is known to those skilled in the art that other methods can also be used in the context of the present invention to reduce an ε–cyclase or its activity or function. For example, the introduction of a dominant-negative variant of an ε–cyclase or of an expression cassette ensuring expression thereof can also be advantageous. Each individual one of

these methods can cause a reduction in the amount of protein, amount of mRNA and/or activity of an ε–cyclase. Combined employment is also conceivable. Further methods are known to those skilled in the art and can comprise the impairment or prevention of processing of the ε–cyclase, of transport of the ε–cyclase or mRNA thereof, inhibition of ribosome attachment, inhibition of RNA splicing, induction of an ε–cyclase RNA-degrading enzyme and/or inhibition of translation elongation or termination.

The individual preferred methods may be described hereinafter by exemplary embodiments:

a) introduction of a double-stranded ε–cyclase ribonucleic acid sequence (ε–cyclase-dsRNA)

The method of gene regulation by means of double-stranded RNA ("double-stranded RNA interference"; dsRNAi) is known and is described, for example, in Matzke MA et al. (2000) Plant Mol Biol 43:401-415; Fire A. et al. (1998) Nature 391:806-811; WO 99/32619; WO 99/53050; WO 00/68374; WO 00/44914; WO 00/44895; WO 00/49035 or WO 00/63364. The processes and methods described in the citations reported are hereby explicitly incorporated by reference into the present application.

"Double-stranded ribonucleic acid sequence" is taken to mean, according to the invention, one or more ribonucleic acid sequences which, owing to complementary sequences, theoretically, for example according to the base pair rules of Watson and Crick and/or in reality, for example on the basis of hybridization experiments, are able, in vitro and/or in vivo, to form double-stranded RNA structures.

It is known to those skilled in the art that the formation of double-stranded RNA structures is an equilibrium state. Preferably, the ratio of double-stranded molecules to corresponding dissociated forms is at least 1 to 10, preferably 1:1, particularly preferably 5:1, most preferably 10:1.

A double-stranded ε–cyclase ribonucleic acid sequence or ε–cyclase-dsRNA is preferably taken to mean an RNA molecule which has a region having double-stranded structure and, in this region, comprises a nucleic acid sequence which

a) is identical to at least a part of the ε-cyclase transcript inherent to the plant and/or

b) is identical to at least a part of the ε-cyclase promoter sequence inherent to the plant.

In the inventive method, therefore to reduce the ε-cyclase activity, preferably an RNA is introduced into the plant, which RNA has a region having double-stranded structure and, in this region, comprises a nucleic acid sequence which

a) is identical to at least a part of the ε-cyclase transcript inherent to the plant and/or

b) is identical to at least a part of the ε-cyclase promoter sequence inherent to the plant.

The term "ε-cyclase transcript" is taken to mean the transcribed part of an ε-cyclase gene which, in addition to the sequence coding for ε-cyclase, also comprises, for example, non-coding sequences, for example also UTRs.

An RNA which "is identical to at least a part of the ε-cyclase promoter sequence inherent to the plant" is preferably taken to mean that the RNA sequence is identical to at least a part of the theoretical transcript of the ε-cyclase promoter sequence, that is to say to the corresponding RNA sequence.

"A part" of the ε-cyclase transcript inherent to the plant or of the ε-cyclase promoter sequence inherent to the plant is taken to mean partial sequences which can range from a few base pairs up to complete sequences of the transcript or of the promoter sequence. The optimum length of the partial sequences can readily be determined by those skilled in the art by routine experiments.

Generally, the length of the partial sequences is at least 10 bases and at most 2 kb, preferably at least 25 bases and at most 1.5 kb, particularly preferably at least 50 bases and at most 600 bases, very particularly preferably at least 100 bases and at most 500, most preferably at least 200 bases or at least 300 bases and at most 400 bases.

Preferably, the partial sequences are sought out in such a manner that a specificity as high as possible is achieved and activities of other enzymes, the reduction of which is not desired, are not reduced. It is therefore advantageous for the partial sequences of the ε-cyclase-dsRNA to select parts of the ε-cyclase transcript and/or partial sequences of the ε-cyclase promoter sequences which do not occur in other activities.

In a particularly preferred embodiment, therefore, the ε-cyclase-dsRNA comprises a sequence which is identical to a part of the plant-inherent ε-cyclase transcript and comprises the 5' end or the 3' end of the plant-inherent nucleic acid coding for an ε-cyclase. In particular, non-translated regions in the 5' or 3' of the transcript are suitable to produce selective double-stranded structures.

The invention further relates to double-stranded RNA molecules (dsRNA molecules) which, on introduction into a plant organism (or a cell, tissue, organ or propagating material derived therefrom), cause the reduction of an ε–cyclase.

A double-stranded RNA molecule for reducing the expression of an ε–cyclase (ε–cyclase-dsRNA) preferably comprises

a) a "sense" RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least one part of a "sense" RNA-ε-cyclase transcript, and

b) an "antisense" RNA strand which is essentially, preferably completely, complementary to the RNA "sense" strand under a).

To transform the plant with an ε–cyclase-dsRNA, preferably a nucleic acid construct is used which is introduced into the plant and which is transcribed in the plant into the ε–cyclase-dsRNA.

Therefore, the present invention also relates to a nucleic acid construct which can be transcribed into

a) a "sense" RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of the "sense" RNA-ε-cyclase transcript, and

b) an "antisense" RNA strand which is essentially, preferably completely, complementary to the RNA sense strand under a).

These nucleic acid constructs are also termed hereinafter expression cassettes or expression vectors.

In relation to the dsRNA molecules, ε–cyclase nucleic acid sequence, or the corresponding transcript, is preferably taken to mean the sequence according to SEQ ID NO: 38 or a part of same.

"Essentially identical" means that the dsRNA sequence can also have insertions, deletions and individual point mutations compared with the ε–cyclase target sequence, and nevertheless causes an efficient reduction of expression. Preferably, the homology is at least 75%, preferably at least 80%, very particularly preferably at least 90%, most preferably 100%, between the "sense" strand of an inhibitory dsRNA and at least a part of the "sense" RNA transcript of an ε–cyclase gene, or between the "antisense" strand and the complementary strand of an ε–cyclase gene.

A 100% sequence identity between dsRNA and an ε–cyclase gene transcript is not absolutely necessary in order to cause efficient reduction of the ε–cyclase expression. Accordingly, there is the advantage that the method is tolerant toward sequence deviations as can be present owing to genetic mutations, polymorphisms or evolutionary divergences. Thus, it is possible, for example, with the dsRNA, which was generated starting from the ε–cyclase sequence of the one organism, to suppress the ε–cyclase expression in another organism. For this purpose, the dsRNA preferably comprises sequence regions of ε–cyclase gene transcripts which correspond to conserved regions. Said conserved regions can be readily derived from sequence comparisons.

Alternatively, an "essentially identical" dsRNA can also be defined as a nucleic acid sequence which is capable of hybridizing with a part of an ε–cyclase gene transcript

(e.g. in 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA at 50°C or 70°C for 12 to 16 h).

"Essentially complementary" means that the "antisense" RNA strand can also have insertions, deletions and also individual point mutations compared with the complement of the "sense" RNA strand. Preferably, the homology is at least 80%, preferably at least 90%, very particularly preferably at least 95%, most preferably 100%, between the "antisense" RNA strand and the complement of the "sense" RNA strand.

In a further embodiment, the ε–cyclase-dsRNA comprises

a) a "sense" RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of the "sense" RNA transcript of the promoter region of an ε-cyclase gene, and

b) an "antisense" RNA strand which is essentially, preferably completely, complementary to the RNA "sense" strand under a).

The corresponding nucleic acid construct which is preferably to be used for transforming the plants comprises

a) a "sense" DNA strand which is essentially identical to at least a part of the promoter region of an ε-cyclase gene, and

b) an "antisense" DNA strand which is essentially, preferably completely, complementary to the DNA "sense" strand under a).

Preferably, the promoter region of an ε-cyclase is taken to mean a sequence according to SEQ ID NO: 47 or a part of same.

To produce the ε–cyclase-dsRNA sequences for reducing the ε-cyclase activity, use is particularly preferably made of the following partial sequences, in particular for *Tagetes erecta*:

SEQ ID NO: 40: sense fragment of the 5'-terminal region of the ε-cyclase

SEQ ID NO: 41: antisense fragment of the 5'-terminal region of the ε–cyclase

SEQ ID NO: 42: sense fragment of the 3'-terminal region of the ε–cyclase

SEQ ID NO: 43: antisense fragment of the 3'-terminal region of the ε–cyclase

SEQ ID NO: 47: sense fragment of the ε–cyclase promoter

SEQ ID NO: 48: antisense fragment of the ε–cyclase promoter

The dsRNA can consist of one or more strands of polyribonucleotides. Obviously, in order to achieve the same purpose, a plurality of individual dsRNA molecules, each of which comprises one of the above-defined ribonucleotide sequence sections, can also be introduced into the cell or the organism.

The double-stranded dsRNA structure can be formed starting from two complementary separate RNA strands or, preferably, starting from a single, self-complementary, RNA strand. In this case, "sense" RNA strand and "antisense" RNA strand are preferably covalently bound to one another in the form of an inverted "repeat".

As described, for example, in WO 99/53050, the dsRNA can also comprise a hairpin structure, by "sense" and "antisense" strand being connected by a linking sequence ("linker"; for example an intron). The self-complementary dsRNA structures are preferred, since they only require the expression of one RNA sequence and always comprise the complementary RNA strands in an equimolar ratio. Preferably, the linking sequence is an intron (for example an intron of the ST-LS1 gene from potato; Vancanneyt GF et al. (1990) Mol Gen Genet 220(2):245-250).

The nucleic acid sequence coding for a dsRNA can contain further elements, for example transcription termination signals or polyadenylation signals.

However, if the dsRNA is directed against the promoter sequence of an ε-cyclase, it preferably does not comprise transcription termination signals or polyadenylation signals. This enables retention of the dsRNA in the nucleus of the cell and prevents distribution of the dsRNA in the entire plant "spreading").

If the two strands of the dsRNA are to be brought together in a cell or plant, this can be achieved, for example, in the following manner:

a) transformation of the cell or plant by a vector which comprises both expression cassettes,

b) cotransformation of the cell or plant using two vectors, the one comprising the expression cassettes with the "sense" strand, the other comprising the expression cassettes with the "antisense" strand.

c) crossing of two individual plant lines, the one comprising the expression cassettes with the "sense" strand, the other comprising the expression cassettes with the "antisense" strand.

The formation of the RNA duplex can be initiated either outside the cell or inside same.

The dsRNA can be synthesized either in vivo or in vitro. For this, a DNA sequence coding for a dsRNA can be placed into an expression cassette under the control of at least one genetic control element (for example a promoter). Polyadenylation is not necessary, likewise, elements for initiating a translation need not be present. Preferably, the expression cassette for the MP-dsRNA is present on the transformation construct or the transformation vector.

In a particularly preferred embodiment, the dsRNA is expressed starting from an expression construct under functional control of a flower-specific promoter, particularly preferably under the control of the promoter described by SEQ ID NO: 28 or a functionally equivalent part of same.

The expression cassettes coding for the "antisense" and/or the "sense" strand of an ε–cyclase-dsRNA, or for the self-complementary strand of the dsRNA are, for this, preferably inserted into a transformation vector and introduced into the plant cell using the methods described below. For the inventive method, a stable insertion into the genome is advantageous.

The dsRNA can be introduced in an amount which makes possible at least one copy per cell. Higher amounts (for example at least 5, 10, 100, 500 or 1000 copies per cell) can if appropriate cause a more efficient reduction.

b) Introduction of an antisense ribonucleic acid sequence of an ε–cyclase (ε–cyclase-antisense RNA)

Methods for reducing a certain protein by "antisense" technology have been described repeatedly, also in plants (Sheehy et al. (1988) Proc Natl Acad Sci USA 85: 8805-8809; US 4,801,340; Mol JN et al. (1990) FEBS Lett 268(2):427-430). The antisense nucleic acid molecule hybridizes with or binds to the cellular mRNA and/or genomic DNA coding for the ε–cyclase to be reduced. This suppresses the transcription and/or translation of the ε–cyclase. The hybridization can be performed in a conventional manner via the formation of a stable duplex or, in the case of genomic DNA, by binding the antisense nucleic acid molecule to the duplex of the genomic DNA by specific interaction in the deep groove of the DNA helix.

An ε–cyclase-antisense RNA can be derived according to the base pair rules of Watson and Crick using the nucleic acid sequence coding for this ε–cyclase, for example the nucleic acid sequence according to SEQ ID NO: 38. The ε–cyclase-antisense RNA can be complementary to the entire transcribed mRNA of the ε–cyclase, be restricted to the coding region, or consist only of an oligonucleotide which is in part complementary to the coding or non-coding sequence of the mRNA. For instance, the oligonucleotide can, for example, be complementary to the region which comprises the start of translation for the ε–cyclase. The ε–cyclase-antisense RNA can have a length of, for example, 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides, but can also be longer and comprise at least 100, 200, 500, 1000, 2000 or 5000 nucleotides. ε–Cyclase-antisense RNAs, in the context of the inventive method, are preferably expressed in a recombinant manner in the target cell.

In a particularly preferred embodiment, the antisense RNA is expressed starting from an expression construct under functional control of a flower-specific promoter, particularly preferably under the control of the promoter described by SEQ ID NO: 28, or a functionally equivalent part of same.

Said expression cassettes can be part of a transformation construct or transformation vector, or else be introduced in the context of a cotransformation.

In a further, preferred embodiment, the expression of an ε–cyclase can be inhibited by nucleotide sequences which are complementary to the regulatory region of an ε–cyclase gene (for example an ε–cyclase promoter and/or enhancer) and form triple-helical structures with the DNA double helix there, so that the transcription of the ε–cyclase gene is reduced. Corresponding methods are described (Helene C (1991) Anticancer Drug Res 6(6):569-84; Helene C et al. (1992) Ann NY Acad Sci 660:27-36; Maher LJ (1992) Bioassays 14(12):807- 815).

In a further embodiment, the ε–cyclase-antisense RNA can be an α-anomeric nucleic acid. Such α-anomeric nucleic acid molecules form specific double-stranded hybrids with complementary RNA in which, in contrast to the conventional β-nucleic acids, the two strands run parallel to one another (Gautier C et al. (1987) Nucleic Acids Res 15:6625-6641).

c) Introduction of an ε–cyclase-antisense RNA combined with a ribozyme

Advantageously, the above-described antisense strategy can be coupled to a ribozyme method. Catalytic RNA molecules or ribozymes can be adapted to any desired target RNA and cleave the phosphodiester backbone at specific positions, which functionally deactivate the target RNA (Tanner NK (1999) FEMS Microbiol Rev 23(3):257-275). The ribozyme is not modified itself as a result, but is able to cleave further target RNA molecules in a similar manner, which gives it the properties of an enzyme. The incorporation of ribozyme sequences into "antisense" RNAs gives precisely these "antisense" RNAs this enzyme-like RNA-cleaving property, and thus increases their efficiency in the inactivation of the target RNA. The production and use of corresponding ribozyme "antisense" RNA molecules is described (inter alia in Haseloff et al. (1988) Nature 334: 585-591); Haselhoff and Gerlach (1988) Nature 334:585-591; Steinecke P et al. (1992) EMBO J 11(4):1525-1530; de Feyter R et al. (1996) Mol Gen Genet. 250(3):329-338).

In this manner, ribozymes (for example "Hammerhead" ribozymes; Haselhoff and Gerlach (1988) Nature 334:585-591) can be used to cleave catalytically the mRNA of

an ε–cyclase to be reduced, and thus prevent translation. The ribozyme technology can increase the efficiency of an antisense strategy. Methods for the expression of ribozymes for reducing certain proteins are described in (EP 0 291 533, EP 0 321 201, EP 0 360 257). In plant cells, ribozyme expression is likewise described (Steinecke P et al. (1992) EMBO J 11(4):1525-1530; de Feyter R et al. (1996) Mol Gen Genet. 250(3):329-338). Suitable target sequences and ribozymes can be determined, for example, as described in "Steinecke P, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds, Academic Press, Inc. (1995), pp. 449-460", by secondary structural calculations of ribozyme and target RNA and also by interaction thereof (Bayley CC et al. (1992) Plant Mol Biol. 18(2):353-361; Lloyd AM and Davis RW et al. (1994) Mol Gen Genet. 242(6):653-657). For example, derivatives of the Tetrahymena L-19 IVS RNA can be constructed which have regions complementary to the mRNA of the ε–cyclase to be suppressed (see also US 4,987,071 and US 5,116,742). Alternatively, such ribozymes can also be identified via a selection process from a library of diverse ribozymes (Bartel D and Szostak JW (1993) Science 261:1411-1418).

d) Introduction of a sense ribonucleic acid sequence of an ε–cyclase (ε–cyclase-sense RNA) for induction of a cosuppression

The expression of an ε–cyclase ribonucleic acid sequence (or a part of same) in the sense orientation can lead to cosuppression of the corresponding ε–cyclase gene. The expression of sense RNA having homology to an endogenous ε–cyclase gene can reduce or switch off the expression of same, in a similar manner to that which has been described for antisense approaches (Jorgensen et al. (1996) Plant Mol Biol 31(5):957-973; Goring et al. (1991) Proc Natl Acad Sci USA 88:1770-1774; Smith et al. (1990) Mol Gen Genet 224:447-481; Napoli et al. (1990) Plant Cell 2:279-289; Van der Krol et al. (1990) Plant Cell 2:291-99). The construct introduced can represent the homologous gene to be reduced entirely or only in part. The possibility of translation is not required. The application of this technology to plants is described (for example Napoli et al. (1990) Plant Cell 2:279-289; in US 5,034,323).

Preferably, the cosuppression is implemented using a sequence which is essentially identical to at least a part of the nucleic acid sequence coding for an ε–cyclase, for example the nucleic acid sequence according to SEQ ID NO: 38. Preferably, the ε–cyclase-sense RNA is selected so that translation of the ε–cyclase or a part of same

cannot occur. For this, for example, the 5'-untranslated or 3'-untranslated region can be selected, or else the ATG start codon can be deleted or mutated.

e) Introduction of DNA- or protein-binding factors against ε–cyclase genes, RNAs or proteins

A reduction of ε–cyclase expression is also possible using specific DNA-binding factors, for example using factors of the zinc finger transcription factor type. These factors attach themselves to the genomic sequence of the endogenous target gene, preferably in the regulatory regions, and cause a reduction of expression. Corresponding methods for producing corresponding factors are described (Dreier B et al. (2001) J Biol Chem 276(31):29466-78; Dreier B et al. (2000) J Mol Biol 303(4):489-502; Beerli RR et al. (2000) Proc Natl Acad Sci USA 97 (4):1495-1500; Beerli RR et al. (2000) J Biol Chem 275(42):32617-32627; Segal DJ and Barbas CF 3rd. (2000) Curr Opin Chem Biol 4(1):34-39; Kang JS and Kim JS (2000) J Biol Chem 275(12):8742-8748; Beerli RR et al. (1998) Proc Natl Acad Sci USA 95(25):14628-14633; Kim JS et al. (1997) Proc Natl Acad Sci USA 94(8):3616-3620; Klug A (1999) J Mol Biol 293(2):215-218; Tsai SY et al. (1998) Adv Drug Deliv Rev 30(1-3):23-31; Mapp AK et al. (2000) Proc Natl Acad Sci USA 97(8):3930-3935; Sharrocks AD et al. (1997) Int J Biochem Cell Biol 29(12):1371-1387; Zhang L et al. (2000) J Biol Chem 275(43):33850-33860).

These factors can be selected using any desired piece of an ε–cyclase gene. Preferably, this section is in the promoter region. For gene suppression, however, it can also be in the region of the coding exons or introns.

In addition, factors can be introduced into a cell which inhibit the ε–cyclase itself. These protein-binding factors can be, for example, aptamers (Famulok M and Mayer G (1999) Curr Top Microbiol Immunol 243:123-36) or antibodies or antibody fragments or single-chain antibodies. The isolation of these factors is described (Owen M et al. (1992) Biotechnology (N Y) 10(7):790-794; Franken E et al. (1997) Curr Opin Biotechnol 8(4):411-416; Whitelam (1996) Trend Plant Sci 1:268-272).

f) Introduction of the viral nucleic acid sequences causing ε–cyclase RNA breakdown, and expression constructs

The ε–cyclase expression can also be effectively achieved by induction of the specific ε–cyclase RNA breakdown by the plant using a viral expression system (Amplikon; Angell SM et al. (1999) Plant J 20(3):357-362). These systems, also termed "VIGS" (virus-induced gene silencing), introduce into the plant by means of viral vectors nucleic acid sequences having homology to the transcript of an ε–cyclase to be reduced. The transcription is then switched off, presumably mediated by plant defense mechanisms against viruses. Corresponding techniques and methods are described (Ratcliff F et al. (2001) Plant J 25(2):237-45; Fagard M and Vaucheret H (2000) Plant Mol Biol 43(2-3):285-93; Anandalakshmi R et al. (1998) Proc Natl Acad Sci USA 95(22):13079-84; Ruiz MT (1998) Plant Cell 10(6):937-46).

Preferably, the VIGS-mediated reduction is implemented using a sequence which is essentially identical to at least a part of the nucleic acid sequence coding for an ε–cyclase, for example the nucleic acid sequence according to SEQ ID NO: 1.

g) Introduction of constructs to generate a loss of function, or a reduction in function of ε–cyclase genes

Those skilled in the art know numerous methods as to how genomic sequences can be specifically modified. These include, in particular, methods such as generating knockout mutants by means of targeted homologous recombination, for example by generating stop codons, shifts in the reading frame etc. (Hohn B and Puchta H (1999) Proc Natl Acad Sci USA 96:8321-8323) or the targeted deletion or inversion of sequences by means of, for example, sequence-specific recombinases or nucleases (see below).

The reduction of ε–cyclase amount, function and/or activity can also be achieved by a targeted insertion of nucleic acid sequences (for example the nucleic acid sequence to be inserted in the context of the inventive method) into the sequence coding for an ε–cyclase (for example by means of intermolecular homologous recombination). In the context of this embodiment, use is preferably made of a DNA construct which comprises at least a part of the sequence of an ε–cyclase gene or neighboring sequences and can thus be specifically recombined with these in the target cell, so that deletion, addition or substitution of at least one nucleotide changes the ε–cyclase gene

in such a manner that the functionality of the ε–cyclase gene is reduced or completely eliminated. The change can also relate to the regulative elements (for example the promoter) of the ε–cyclase gene, so that the coding sequence remains unchanged, however expression (transcription and/or translation) is stopped and reduced. In the conventional homologous recombination, the sequence to be inserted is flanked at its 5'- and/or 3'-end by further nucleic acid sequences (A' or B') which have sufficient length and homology to corresponding sequences of the ε–cyclase gene (A and B) to enable homologous recombination. The length is generally in a range from several hundred bases up to several kilo bases (Thomas KR and Capecchi MR (1987) Cell 51:503; Strepp et al. (1998) Proc Natl Acad Sci USA 95(8):4368-4373). For homologous recombination, the plant cell having the recombination construct is transformed using the methods described below and successfully recombined clones are selected on the basis of the consequent inactivated ε–cyclase.

In a further preferred embodiment, the efficiency of recombination is increased by combination with methods which promote homologous recombination. Such methods are described and comprise, for example, the expression of proteins such as RecA, or treatment with PARP inhibitors. It has been found that the intrachromosomal homologous recombination in tobacco plants can be increased by using PARP inhibitors (Puchta H et al. (1995) Plant J 7:203-210). By using these inhibitors, the rate of homologous recombination in the recombination constructs after induction of the sequence-specific DNA double strand break, thus the efficiency of deletion of the transgenic sequences, can be further increased. Various PARP inhibitors can be used for this. Those which are preferably comprised are inhibitors such as 3-aminobenzamide, 8-hydroxy-2-methylquinazolin-4-one (NU1025), 1,11b-dihydro-[2H]benzopyrano-[4,3,2-de]isoquinolin-3-one (GPI 6150), 5-aminoisoquinolinone, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1-(2H)isoquinolinone, or the substances described in WO 00/26192, WO 00/29384, WO 00/32579, WO 00/64878, WO 00/68206, WO 00/67734, WO 01/23386 and WO 01/23390.

Other suitable methods are the introduction of nonsense mutations into endogenous marker protein genes, for example by means of introducing RNA/DNA oligonucleotides into the plant (Zhu et al. (2000) Nat Biotechnol 18(5):555-558), or generating knockout mutants using, for example, T-DNA mutagenesis (Koncz et al., Plant Mol. Biol. 1992, 20(5):963-976). Point mutations can also be generated by means of DNA-RNA hybrids,

which are also known as "chimeraplasty" (Cole-Strauss et al. (1999) Nucl Acids Res 27(5):1323-1330; Kmiec (1999) Gene therapy American Scientist 87(3):240-247).

The methods of dsRNAi, cosuppression by means of sense RNA, and "VIGS" ("virus-induced gene silencing") are also termed "post-transcriptional gene silencing" (PTGS) or "transcriptional gene silencing" (TGS). PTGS/TGS methods are particularly advantageous because the requirements for homology between the marker protein gene to be decreased and the transgenically expressed sense or dsRNA nucleic acid sequence are less than, for example, in the case of a classic antisense approach. Thus, using the marker protein nucleic acid sequences from one species, the expression of homologous marker protein proteins in other species can also be effectively reduced without requiring the isolation and structural elucidation of the marker protein homologs occurring there. This considerably reduces the workload.

In a particularly preferred embodiment of the inventive method, the ε-cyclase activity is reduced compared with the wild type by:

a) introducing at least one double-stranded ε-cyclase ribonucleic acid sequence or an expression cassette ensuring expression thereof, or expression cassettes, in plants and/or

b) introducing at least one ε-cyclase antisense ribonucleic acid sequence, or an expression cassette ensuring expression thereof, into plants.

In a very particularly preferred embodiment, the ε-cyclase activity is reduced compared with the wild type by introducing into plants at least one double-stranded ε-cyclase ribonucleic acid sequence or an expression cassette ensuring expression thereof, or expression cassettes.

In a preferred embodiment, genetically modified plants are used which have in flowers the lowest expression rate of an ε-cyclase.

This is preferably achieved by reducing the ε-cyclase activity in a flower-specific manner, particularly preferably in a flower-leaf-specific manner.

In the above-described particularly preferred embodiment, this is achieved by the transcription of the ε–cyclase-dsRNA sequences being formed under control of a flower-specific promoter or, still more preferably, under the control of a flower-leaf-specific promoter.

In a further preferred embodiment, plants are cultivated which, in addition, compared with the wild type have an increased activity of at least one of the activities selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate-synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity.

HMG-CoA reductase activity is taken to mean the enzyme activity of an HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme-A reductase).

A HMG-CoA reductase is taken to mean a protein which has the enzymatic activity to convert 3-hydroxy-3-methylglutaryl coenzyme-A to mevalonate.

Accordingly, HMG-CoA reductase activity is taken to mean the amount of 3-hydroxy-3-methylglutaryl coenzyme-A converted, or amount of mevalonate formed, in a defined time by the protein HMG-CoA reductase.

With an elevated HMG-CoA reductase activity compared with the wild type, therefore, the amount of 3-hydroxy-3-methylglutaryl coenzyme-A converted, or the amount of mevalonate formed, is increased in a defined time by the protein HMG-CoA reductase compared with the wild type.

Preferably, this increase in HMG-CoA reductase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the HMG-CoA reductase activity of the wild type. HMG-CoA reductase activity is taken to mean the enzyme activity of an HMG-CoA reductase.

The HMG-CoA reductase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

The HMG-CoA reductase activity can be measured in accordance with published descriptions (e.g. Schaller, Grausem, Benveniste, Chye, Tan, Song and Chua, Plant Physiol. 109 (1995), 761-770; Chappell, Wolf, Proulx, Cuellar and Saunders, Plant Physiol. 109 (1995) 1337-1343). Plant tissue can be homogenized and extracted in cold buffer (100 mM potassium phosphate (pH 7.0), 4 mM $MgCl_2$, 5 mM DTT). The homogenate is centrifuged at 10 000 g for 15 minutes at 4°C. The supernatant is thereafter centrifuged again at 100 000 g for 45-60 minutes. The HMG-CoA reductase activity is determined in the supernatant and in the pellet of the microsomal fraction (after resuspension in 100 mM potassium phosphate (pH 7.0) and 50 mM DTT). Aliquots of the solution and the suspension (the protein content of the suspension is equivalent to about 1-10 μg) are incubated at 30°C for 15-60 minutes in 100 mM potassium phosphate buffer (pH 7.0 comprising 3 mM NADPH and 20 μM (^{14}C)HMG-CoA (58 μCi/μM), ideally in a volume of 26 μl. The reaction is terminated by adding 5 μl of mevalonate lactone (1 mg/ml) and 6 N HCl. After addition, the mixture is incubated at room temperature for 15 minutes. The (^{14}C) mevalonate formed in the reaction is quantified by adding 125 μl of a saturated potassium phosphate solution (pH 6.0) and 300 μl of ethyl acetate. The mixture is mixed well and centrifuged. The radioactivity can be determined by measuring scintillation.

(E)-4-Hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, also termed lytB or IspH, is taken to mean the enzyme activity of an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase.

An (E)-4-Hydroxy-3-methylbut-2-enyl-diphosphate reductase is taken to mean a protein which has the enzymatic activity to convert (E)-4-hydroxy-3-methylbut-2-enyl diphosphate to isopentenyl diphosphate and dimethylallyl diphosphates.

Accordingly, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity is taken to mean the amount of (E)-4-hydroxy-3-methylbut-2-enyl diphosphate converted, or amount of isopentenyl diphosphate and/or dimethylallyl diphosphate formed, in a defined time by the protein (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase.

In the event of an elevated (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity compared with the wild type, thus, compared with the wild type, the amount of (E)-4-hydroxy-3-methylbut-2-enyl diphosphate converted or the amount of isopentenyl diphosphate and/or dimethylallyl diphosphate formed is elevated in a defined time by the protein (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase.

Preferably, this increase in (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity of the wild type.

The (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

The (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity can be determined via immunological detection. The production of specific antibodies has

been described by Rohdich and colleagues (Rohdich, Hecht, Gärtner, Adam, Krieger, Amslinger, Arigoni, Bacher and Eisenreich: Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein, Natl. Acad. Natl. Sci. USA 99 (2002), 1158-1163). For the determination of the catalytic activity, Altincicek and colleagues (Altincicek, Duin, Reichenberg, Hedderich, Kollas, Hintz, Wagner, Wiesner, Beck and Jomaa: LytB protein catalyzes the terminal step of the 2-C-methyl-D-erythritol-4-phosphate pathway of isoprenoid biosynthesis; FEBS Letters 532 (2002) 437-440) describe an in vitro system which follows the reduction of (E)-4-hydroxy-3-methylbut-2-enyl diphosphate to isopentenyl diphosphate and dimethylallyl diphosphate.

1-Deoxy-D-xylose-5-phosphate synthase activity is taken to mean the enzyme activity of a 1-deoxy-D-xylose-5-phosphate synthase.

A 1-deoxy-D-xylose-5-phosphate synthase is taken to mean a protein which has the enzymatic activity to convert hydroxyethyl-ThPP and glyceraldehyde-3-phosphate to 1-deoxy-D-xylose-5-phosphate.

Accordingly, 1-deoxy-D-xylose-5-phosphate synthase activity is taken to mean the amount of hydroxyethyl-ThPP and/or glyceraldehyde-3-phosphate converted, or amount of 1-deoxy-D-xylose-5-phosphate formed in a defined time by the protein 1-deoxy-D-xylose-5-phosphate synthase.

In the event of an elevated 1-deoxy-D-xylose-5-phosphate synthase activity compared with the wild type, thus, the amount of hydroxyethyl-ThPP and/or glyceraldehyde-3-phosphate converted, or the amount of 1-deoxy-D-xylose-5-phosphate formed is elevated in a defined time by the protein 1-deoxy-D-xylose-5-phosphate synthase compared with the wild type.

Preferably, this elevation in 1-deoxy-D-xylose-5-phosphate synthase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the 1-deoxy-D-xylose-5-phosphate synthase activity of the wild type.

The 1-deoxy-D-xylose-5-phosphate synthase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

The reaction solution (50-200 µl) for determination of D-1-deoxyxylulose-5-phosphate synthase activity (DXS) consists of 100 mM Tris-HCl (pH 8.0), 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 mM ATP, 1 mM thiamine diphosphate, 0.1% Tween 60, 1 mM potassium fluoride, 30 µM (2-^{14}C) pyruvate (0.5 µCi), 0.6 mM DL-glyceraldehyde-3-phosphate. The plant extract is incubated in the reaction solution at 37°C for 1 to 2 hours. Thereafter, the reaction is terminated by heating to 80°C for 3 minutes. After centrifugation at 13 000 revolutions per minute for 5 minutes, the supernatant is evaporated, the remainder is resuspended in 50 µl of methanol, applied to a TLC plate for thin-layer chromatography (Silica-Gel 60, Merck, Darmstadt) and separated in n-propyl alcohol/ethyl acetate/water (6:1:3; v/v/v). Radioactively labeled D-1-deoxyxylulose-5-phosphate (or D-1-deoxyxylulose) separates from (2-^{14}C) pyruvate. The quantification is performed using a scintillation counter. The method has been described in Harker and Bramley (FEBS Letters 448 (1999) 115-119). Alternatively, a fluorometric assay for determining the DXS synthase activity has been described by Querol and colleagues (Analytical Biochemistry 296 (2001) 101-105).

1-Deoxy-D-xylose-5-phosphate reductoisomerase activity is taken to mean the enzyme activity of a 1-deoxy-D-xylose-5-phosphate reductoisomerase.

A 1-deoxy-D-xylose-5-phosphate reductoisomerase is taken to mean a protein which has the enzymatic activity to convert 1-deoxy-D-xylose-5-phosphate to β-carotene.

Accordingly, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity is taken to mean the amount of 1-deoxy-D-xylose-5-phosphate converted or amount of isopentenyl diphosphate formed in a defined time by the protein 1-deoxy-D-xylose-5-phosphate reductoisomerase.

In the event of an elevated 1-deoxy-D-xylose-5-phosphate reductoisomerase activity compared with the wild type, thus, the amount of 1-deoxy-D-xylose-5-phosphate converted, or the amount of isopentenyl diphosphate formed, is elevated in a defined time by the protein 1-deoxy-D-xylose-5-phosphate reductoisomerase compared with the wild type.

Preferably, this elevation in 1-deoxy-D-xylose-5-phosphate reductoisomerase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the 1-deoxy-D-xylose-5-phosphate reductoisomerase activity of the wild type.

The 1-deoxy-D-xylose-5-phosphate reductoisomerase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

The activity of D-1-deoxyxylulose-5-phosphate reductoisomerase (DXR) is measured in a buffer consisting of 100 mM Tris-HCl (pH 7.5), 1 mM $MnCl_2$, 0.3 mM NADPH and 0.3 mM 1-deoxy-D-xylulose-4-phosphate, which can be synthesized enzymatically, for example (Kuzuyama, Takahashi, Watanabe and Seto: Tetrahedon letters 39 (1998) 4509-4512). The reaction is started by addition of the plant extract. The reaction volume can typically be from 0.2 to 0.5 ml; the incubation is performed at 37°C over

30-60 minutes. During this time, the oxidation of NADPH is followed photometrically at 340 nm.

Isopentenyl-diphosphate Δ-isomerase activity is taken to mean the enzyme activity of an isopentenyl-diphosphate Δ-isomerase.

An isopentenyl-diphosphate D-isomerase is taken to mean a protein which has the enzymatic activity to convert isopentenyl diphosphate to dimethylallyl phosphate.

Accordingly, isopentenyl-diphosphate D-isomerase activity is taken to mean the amount of isopentenyl diphosphate converted or amount of dimethylallyl phosphate formed in a defined time by the protein isopentenyl-diphosphate D-isomerase.

In the event of an elevated isopentenyl-diphosphate D-isomerase activity compared with the wild type, thus the amount of isopentenyl diphosphate converted or the amount of dimethylallyl phosphate formed is elevated in a defined time by the protein isopentenyl-diphosphate D-isomerase compared with the wild type.

Preferably, this elevation in isopentenyl-diphosphate Δ-isomerase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the isopentenyl-diphosphate Δ-isomerase activity of the wild type.

The isopentenyl-diphosphate Δ-isomerase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton

X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

The isopentenyl-diphosphate isomerase (IPP isomerase) activity can be determined according to the method published by Fraser and colleagues (Fraser, Römer, Shipton, Mills, Kiano, Misawa, Drake, Schuch and Bramley: Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner; Proc. Natl. Acad. Sci. USA 99 (2002), 1092-1097, based on Fraser, Pinto, Holloway and Bramley, Plant Journal 24 (2000), 551-558). For enzyme assays, incubations are carried out using 0.5 µCi (1-^{14}C) IPP (isopentenyl pyrophosphate) (56 mCi/mmol, Amersham plc) as substrate in 0.4 M Tris-HCl (pH 8.0) comprising 1 mM DTT, 4 mM $MgCl_2$, 6 mM $MnCl_2$, 3 mM ATP, 0.1% Tween 60, 1 mM potassium fluoride in a volume of about 150-500 µl. Extracts are mixed with buffer (for example in a ratio of 1:1) and incubated at 28°C for at least 5 hours. Thereafter, about 200 µl of methanol are added and an acid hydrolysis is carried out at 37°C for about 1 hour by adding concentrated hydrochloric acid (final concentration 25%). Then, a twice-repeated extraction (in each case 500 µl) with petroleum ether (admixed with 10% of diethyl ether) is carried out. The radioactivity is determined in an aliquot of the hyperphase using a scintillation counter. The specific enzyme activity can be determined with a short incubation of 5 minutes, since short reaction times suppress the formation of reaction by-products (see Lützow and Beyer: The isopentenyl-diphosphate Δ-isomerase and its relation to the phytoene synthase complex in daffodil chromoplasts; Biochim. Biophys. Acta 959 (1988), 118-126).

Geranyl-diphosphate synthase activity is taken to mean the enzyme activity of a geranyl-diphosphate synthase.

A geranyl-diphosphate synthase is taken to mean a protein which has the enzymatic activity to convert isopentenyl diphosphate and dimethylallyl phosphate to geranyl diphosphate.

Accordingly, geranyl-diphosphate synthase activity is taken to mean the amount of isopentenyl diphosphate and/or dimethylallyl phosphate converted, or amount of geranyl diphosphate formed by the protein geranyl-diphosphate synthase in a defined time.

In the case of an elevated geranyl-diphosphate synthase activity compared with the wild type, thus, the amount of isopentenyl diphosphate and/or dimethylallyl phosphate converted, or the amount of geranyl diphosphate formed, is elevated by the protein geranyl-diphosphate synthase in a defined time compared with the wild type.

Preferably, this elevation in geranyl-diphosphate synthase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the geranyl-diphosphate synthase activity of the wild type.

The geranyl-diphosphate synthase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

The geranyl-diphosphate synthase (GPP synthase) activity can be determined in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2 mM DTT, 1 mM ATP, 0.2% Tween-20, 5 µM (^{14}C) IPP and 50 µM DMAPP (dimethylallyl pyrophosphate) after addition of plant extract (according to Bouvier, Suire, d'Harlingue, Backhaus and Camara: Molecular cloning of geranyl diphosphate synthase and compartmentation of monoterpene synthesis in plant cells, Plant Journal 24 (2000) 241-252). After the incubation at 37°C for, for example, 2 hours, the reaction products are dephosphorylated (according to Koyama, Fuji and Ogura: Enzymatic hydrolysis of polyprenyl pyrophosphate, Methods Enzymol. 110 (1985), 153-155) and analyzed by means of thin-layer chromatography and measurement of the radioactivity incorporated (Dogbo, Bardat, Quennemet and Camara: Metabolism of plastid terpenoids: In vitro

inhibition of phytoene synthesis by phenethyl pyrophosphate derivates, FEBS Letters 219 (1987) 211-215).

Farnesyl-diphosphate synthase activity is taken to mean the enzyme activity of a farnesyl-diphosphate synthase.

A farnesyl-diphosphate synthase is taken to mean a protein which has the enzymatic activity to convert geranyl diphosphates and isopentenyl diphosphate into farnesyl diphosphate.

Accordingly, farnesyl-diphosphate synthase activity is the amount of geranyl diphosphates and/or isopentenyl diphosphate converted, or amount of farnesyl diphosphate formed, by the protein farnesyl-diphosphate synthase in a defined time.

In the event of an elevated farnesyl-diphosphate synthase activity compared with the wild type, thus the amount of geranyl diphosphate and/or isopentenyl diphosphate converted, or the amount of farnesyl diphosphate formed, is elevated by the protein farnesyl-diphosphate synthase in a defined time.

Preferably, this elevation in farnesyl-diphosphate synthase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the farnesyl-diphosphate synthase activity of the wild type.

The farnesyl-diphosphate synthase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

The farnesyl-pyrophosphate synthase (FPP synthase) activity can be determined according to a procedure by Joly and Edwards (Journal of Biological Chemistry 268 (1993), 26983-26989). According to this, the enzyme activity is assayed in a buffer of 10 mM HEPES (pH 7.2), 1 mM $MgCl_2$, 1 mM dithiothreitol, 20 µM geranyl pyrophosphate and 40 µM (1-^{14}C) isopentenyl pyrophosphate (4 Ci/mmol). The reaction mixture is incubated at 37°C; the reaction is terminated by adding 2.5 N HCl (in 70% ethanol comprising 19 µg/ml of farnesol). The reaction products are thus hydrolyzed by acid hydrolysis at 37°C within 30 minutes. By adding 10% NaOH, the mixture is neutralized, and is extracted by shaking with hexane. An aliquot of the hexane phase can be measured by means of a scintillation counter to determine the radioactivity incorporated.

Alternatively, after incubation of plant extract and radioactively labeled IPP, the reaction products can be separated by thin-layer chromatography (Silica-Gel SE60, Merck) in benzene/methanol (9:1). Radioactively labeled products are eluted and the radioactivity is determined (according to Gaffe, Bru, Causse, Vidal, Stamitti-Bert, Carde and Gallusci: LEFPS1, a tomato farnesyl pyrophosphate gene highly expressed during early fruit development; Plant Physiology 123 (2000) 1351-1362).

Geranylgeranyl-diphosphate synthase activity is taken to mean the enzyme activity of a geranylgeranyl-diphosphate synthase.

A geranylgeranyl-diphosphate synthase is taken to mean a protein which has the enzymatic activity to convert farnesyl diphosphate and isopentenyl diphosphate into geranylgeranyl diphosphate.

Accordingly, geranylgeranyl-diphosphate synthase activity is taken to mean the amount of farnesyl diphosphate and/or isopentenyl diphosphate converted, or amount of geranylgeranyl diphosphate formed, by the protein geranylgeranyl-diphosphate synthase in a defined time.

In the case of an elevated geranylgeranyl-diphosphate synthase activity compared with the wild type, thus the amount of farnesyl diphosphate and/or isopentenyl diphosphate converted, or the amount of geranylgeranyl diphosphate formed, is elevated by the

protein geranylgeranyl-diphosphate synthase in a defined time compared with the wild type.

Preferably, this elevation in geranylgeranyl-diphosphate synthase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the β-cyclase activity of the wild type.

The geranylgeranyl-diphosphate synthase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

Activity of the geranylgeranyl-pyrophosphate synthase (GGPP synthase) can be assayed according to the method described by Dogbo and Camara (in Biochim. Biophys. Acta 920 (1987), 140-148: Purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthase from Capsicum chromoplasts by affinity chromatography). For this, plant extract is added to a buffer (50 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM dithiothreitol, (1-^{14}C) IPP (0.1 μCi, 10 μM), 15 μM DMAPP, GPP or FPP) having a total volume of about 200 μl. The incubation can be carried out at 30°C for 1-2 hours (or longer). The reaction is terminated by adding 0.5 ml of ethanol and 0.1 ml of 6N HCl. After incubation at 37°C for 10 minutes, the reaction mixture is neutralized with 6N NaOH, mixed with 1 ml of water and extracted by shaking with 4 ml of diethyl ether. The radioactivity is determined in an aliquot (e.g. 0.2 ml) of the ether phase by means of scintillation counting. Alternatively, after acid hydrolysis, the radioactively labeled prenyl alcohols can be extracted by shaking in ether and separated by HPLC (25 cm column Spherisorb ODS-1, 5 μm;

elution with methanol/water (90:10; v/v) at a flow rate of 1 ml/min) and quanitified by means of a radioactivity monitor (in accordance with Wiedemann, Misawa and Sandmann: Purification and enzymatic characterization of the geranylgeranyl pyrophosphate synthase from Erwinia uredovora after expression in Escherichia coli).

Phytoene synthase activity is taken to mean the enzyme activity of a phytoene synthase.

A phytoene synthase is taken to mean a protein which has the enzymatic activity of converting a terminal linear residue of lycopene into a β-ionone ring.

In particular, a phytoene synthase is taken to mean a protein which has the enzymatic activity to convert geranylgeranyl diphosphate into phytoene.

Accordingly, phytoene synthase activity is taken to mean the amount of geranylgeranyl diphosphate converted, or amount of phytoene formed, by the protein phytoene synthase in a defined time.

In the case of an elevated phytoene synthase activity compared with the wild type, thus, the amount of geranylgeranyl diphosphate converted, or the amount of phytoene formed, is elevated by the protein phytoene synthase in a defined time compared with the wild type.

Preferably, this elevation of phytoene synthase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the phytoene synthase activity of the wild type.

The phytoene synthase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-

KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

Phytoene synthase (PSY) activity can be determined by the method published by Fraser and colleagues (Fraser, Romer, Shipton, Mills, Kiano, Misawa, Drake, Schuch and Bramley: Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner; Proc. Natl. Acad. Sci. USA 99 (2002), 1092-1097, based on Fraser, Pinto, Holloway and Bramley, Plant Journal 24 (2000) 551-558). For enzyme assays, incubations with (^{3}H) geranylgeranyl pyrophosphate (15 mCi/mM, American Radiolabeled Chemicals, St. Louis) as substrate are carried out in 0.4 M Tris-HCl (pH 8.0) comprising 1 mM DTT, 4 mM $MgCl_2$, 6 mM $MnCl_2$, 3 mM ATP, 0.1% Tween 60, 1 mM potassium fluoride. Plant extracts are mixed with buffer, for example 295 µl of buffer with extract in a total volume of 500 µl. Incubation is performed for at least 5 hours at 28°C. Then, phytoene is extracted by shaking twice (each time 500 µl) with chloroform. The radioactively labeled phytoene formed during the reaction is separated by means of thin-layer chromatography on silica plates in methanol/water (95:5; v/v). Phytoene can be identified on the silica plates in an iodine-enriched atmosphere (by heating a few iodine crystals). A phytoene standard serves as reference. The amount of radioactively labeled product is determined by measurement in the scintillation counter. Alternatively, phytoene can also be quantified by means of HPLC which is provided with a radioactivity detector (Fraser, Albrecht and Sandmann: Development of high performance liquid chromatographic systems for the separation of radiolabeled carotenes and precursors formed in specific enzymatic reactions; J. Chromatogr. 645 (1993) 265-272).

Phytoene desaturase activity is taken to mean the enzyme activity of a phytoene desaturase.

A phytoene desaturase is taken to mean a protein which has the enzymatic activity to convert phytoene into phytofluene and/or phytofluene into ζ-carotene (zeta-carotene).

Accordingly, phytoene desaturase activity is taken to mean the amount of phytoene or phytofluene converted or the amount of phytofluene or ζ-carotene formed by the protein phytoene desaturase in a defined time.

In the case of an elevated phytoene desaturase activity compared with the wild type, thus the amount of phytoene or phytofluene converted or the amount of phytofluene or ζ-carotene is elevated formed by the protein phytoene desaturase in a defined time compared with the wild type.

Preferably, this elevation in phytoene desaturase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the phytoene desaturase activity of the wild type.

The phytoene desaturase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

The activity of phytoene desaturase (PDS) can be assayed by the incorporation of radioactively labeled (^{14}C) phytoene into unsaturated carotenes (as reported by Römer, Fraser, Kiano, Shipton, Misawa, Schuch and Bramley: Elevation of the provitamin A content of transgenic tomato plants; Nature Biotechnology 18 (2000) 666-669). Radioactively labeled phytoenes can be synthesized as described by Fraser (Fraser, De la Rivas, Mackenzie, Bramley: Phycomyces blakesleanus CarB mutants: their use in assays of phytoene desaturase; Phytochemistry 30 (1991), 3971-3976). Membranes of plastids of the target tissue can be incubated with 100 mM MES buffer (pH 6.0) comprising 10 mM $MgCl_2$ and 1 mM dithiothreitol in a total volume of 1 ml. (^{14}C) Phytoene dissolved in acetone (about 100 000 disintegrations/minute for one incubation in each case) is added, in which case the acetone concentration should not exceed 5% (v/v). This mixture is incubated at 28°C for about 6 to 7 hours in the dark

with shaking. Thereafter, pigments are extracted three times with about 5 ml of petroleum ether (admixed with 10% diethyl ether) and separated by means of HPLC and quantified.

Alternatively, the activity of the phytoene desaturase can be assayed as reported by Fraser et al. (Fraser, Misawa, Linden, Yamano, Kobayashi and Sandmann: Expression in Escherichia coli, purification, and reactivation of the recombinant Erwinia uredovora phytoene desaturase, Journal of Biological Chemistry 267 (1992), 19891-19895).

Zeta-carotene desaturase activity is taken to mean the enzyme activity of a zeta-carotene desaturase.

A zeta-carotene desaturase is taken to mean a protein which has the enzymatic activity to convert ζ-carotene into neurosporin and/or neurosporin into lycopene.

Accordingly, zeta-carotene desaturase activity is taken to mean the amount of ζ-carotene or neurosporin converted, or amount of neurosporin or lycopene formed, by the protein zeta-carotene desaturase in a defined time.

In the event of an elevated zeta-carotene desaturase activity compared with the wild type, thus the amount of ζ-carotene or neurosporin converted, or the amount of neurosporin or lycopene formed, is elevated by the protein zeta-carotene desaturase in a defined time compared with the wild type.

Preferably, this elevation in zeta-carotene desaturase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the zeta-carotene desaturase activity of the wild type.

The zeta-carotene desaturase activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear

range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

Analyses for determining the ξ-carotene desaturase (ZDS desaturase) can be carried out in 0.2 M potassium phosphate (pH 7.8, buffer volume about 1 ml). The analytical method for this was published by Breitenbach and colleagues (Breitenbach, Kuntz, Takaichi and Sandmann: Catalytic properties of an expressed and purified higher plant type ξ-carotene desaturase from Capsicum annuum; European Journal of Biochemistry. 265(1):376-383, 1999 Oct.). Each analytical assay mix comprises 3 mg of phosphytidylcholine which is suspended in 0.4 M potassium phosphate buffer (pH 7.8), 5 µg of ξ-carotene or neurosporenes, 0.02% butylhydroxytoluene, 10 µl of decylplastoquinone (1 mM methanolic stock solution) and plant extract. The volume of the plant extract must be adapted to the amount of ZDS desaturase activity present in order to make quantifications in a linear range of measurement possible. Incubations are typically performed for about 17 hours with vigorous shaking (200 rpm) at about 28°C in the dark. Carotenoids are extracted by addition of 4 ml of acetone at 50°C for 10 minutes with shaking. From this mixture, the carotenoids are transferred to a petroleum ether phase (comprising 10% diethyl ether). The diethyl ether/petroleum ether phase is evaporated under nitrogen, the carotenoids are redissolved in 20 µl and separated and quantified by means of HPLC.

crtISO activity is taken to mean the enzyme activity of a crtISO protein.

A crtISO protein is taken to mean a protein which has the enzymatic activity to convert 7,9,7',9'-tetra-cis-lycopene into all-trans-lycopene.

Accordingly, crtISO activity is taken to mean the amount of 7,9,7',9'-tetra-cis-lycopene converted or amount of all-trans-lycopene formed by the protein b-cyclase in a defined time.

In the event of an elevated crtISO activity compared with the wild type, thus the amount of 7,9,7',9'-tetra-cis-lycopene converted, or the amount of all-trans-lycopene formed, is elevated by the crtISO protein in a defined time compared with the wild type.

Preferably, this elevation in crtISO activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably at least 100%, more preferably at least 300%, still more preferably at least 500%, in particular at least 600%, of the crtISO activity of the wild type.

The crtISO activity in inventive genetically modified plants and in wild type or reference plants is preferably determined under the following conditions:

Frozen plant material is homogenized by intensive grinding in a mortar in liquid nitrogen and extracted with extraction buffer in a ratio of from 1:1 to 1:20. The respective ratio depends on the enzyme activities in the plant material available, so that determination and quantification of the enzyme activities is possible within the linear range of measurement. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. Shortly before the extraction, 2 mM DTT and 0.5 mM PMSF are added.

FtsZ activity is taken to mean the physiological activity of an FtsZ protein.

An FtsZ protein is taken to mean a protein which has activity promoting cell division and plastid division and has homologies to tubulin proteins.

MinD activity is taken to mean the physiological activity of a MinD protein.

A MinD protein is taken to mean a protein which has a multifunctional role in cell division. It is a membrane-associated ATPase and, within the cell, can show an oscillating motion from pole to pole.

Furthermore, the increase in activity of enzymes of the non-mevalonate pathway can lead to a further increase in the desired ketocarotenoid end product. Examples therefor are 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase and 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase. By modifying the gene expression of the corresponding genes, the activity of said enzymes can be increased. The modified concentrations of the relevant proteins can be detected in a standard manner by means of antibodies and corresponding blotting

techniques. The increase in HMG-CoA reductase activity and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity and/or 1-deoxy-D-xylose-5-phosphate synthase activity and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase activity and/or isopentenyl-diphosphate Δ-isomerase activity and/or geranyl-diphosphate synthase activity and/or farnesyl-diphosphate synthase activity and/or geranylgeranyl-diphosphate synthase activity and/or phytoene synthase activity and/or phytoene desaturase activity and/or zeta-carotene desaturase activity and/or crtISO activity and/or FtsZ activity and/or MinD activity can be achieved by various ways, for example by switching off restricting regulatory mechanisms at the expression and protein level, or by increasing gene expression of nucleic acids coding for an HMG-CoA reductase and/or nucleic acids coding for an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or nucleic acids coding for a 1-deoxy-D-xylose-5-phosphate synthase and/or nucleic acids coding for a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or nucleic acids coding for an isopentenyl-diphosphate Δ-isomerase and/or nucleic acids coding for a geranyl-diphosphate synthase and/or nucleic acids coding for a farnesyl-diphosphate synthase and/or nucleic acids coding for a geranylgeranyl-diphosphate synthase and/or nucleic acids coding for a phytoene synthase and/or nucleic acids coding for a phytoene desaturase and/or nucleic acids coding for a zeta-carotene desaturase and/or nucleic acids coding for a crtISO protein and/or nucleic acids coding for a FtsZ protein and/or nucleic acids coding for a MinD protein compared with the wild type.

The gene expression of the nucleic acids coding for an HMG-CoA reductase and/or nucleic acids coding for an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or nucleic acids coding for a 1-deoxy-D-xylose-5-phosphate synthase and/or nucleic acids coding for a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or nucleic acids coding for an isopentenyl-diphosphate Δ-isomerase and/or nucleic acids coding for a geranyl-diphosphate synthase and/or nucleic acids coding for a farnesyl-diphosphate synthase and/or nucleic acids coding for a geranylgeranyl-diphosphate synthase and/or nucleic acids coding for a phytoene synthase and/or nucleic acids coding for a phytoene desaturase and/or nucleic acids coding for a zeta-carotene desaturase and/or nucleic acids coding for a crtISO protein and/or nucleic acids coding for an FtsZ protein and/or nucleic acids coding for a MinD protein can likewise be increased compared with the wild type by various ways, for example by inducing the HMG-CoA reductase gene and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate

reductase gene and/or 1-deoxy-D-xylose-5-phosphate synthase gene and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase gene and/or isopentenyl-diphosphate Δ-isomerase gene and/or geranyl-diphosphate synthase gene and/or farnesyl-diphosphate synthase gene and/or geranylgeranyl-diphosphate synthase gene and/or phytoene synthase gene and/or phytoene desaturase gene and/or zeta-carotene desaturase gene and/or crtISO gene and/or FtsZ gene and/or MinD gene by activators or by introducing one or more copies of the HMG-CoA reductase gene and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase gene and/or 1-deoxy-D-xylose-5-phosphate synthase gene and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase gene and/or isopentenyl-diphosphate Δ-isomerase gene and/or geranyl-diphosphate synthase gene and/or farnesyl-diphosphate synthase gene and/or geranylgeranyl-diphosphate synthase gene and/or phytoene synthase gene and/or phytoene desaturase gene and/or zeta-carotene desaturase gene and/or crtISO gene and/or FtsZ gene and/or MinD gene, also by introducing at least one nucleic acid coding for an HMG-CoA reductase and/or at least one nucleic acid coding for a (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or at least one nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate synthase and/or at least one nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or at least one nucleic acid coding for an isopentenyl-diphosphate Δ-isomerase and/or at least one nucleic acid coding for a geranyl-diphosphate synthase and/or at least one nucleic acid coding for a farnesyl-diphosphate synthase and/or at least one nucleic acid coding for a geranylgeranyl-diphosphate synthase and/or at least one nucleic acid coding for a phytoene synthase and/or at least one nucleic acid coding for a phytoene desaturase and/or at least one nucleic acid coding for a zeta-carotene desaturase and/or at least one nucleic acid coding for a crtISO protein and/or at least one nucleic acid coding for an FtsZ protein and/or at least one nucleic acid coding for a MinD protein into the plant.

Elevation of the gene expression of a nucleic acid coding for an HMG-CoA reductase and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or 1-deoxy-D-xylose-5-phosphate synthase and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or isopentenyl-diphosphate Δ-isomerase and/or geranyl-diphosphate synthase and/or farnesyl-diphosphate synthase and/or geranylgeranyl-diphosphate synthase and/or phytoene synthase and/or phytoene desaturase and/or zeta-carotene desaturase and/or a crtISO protein and/or FtsZ protein and/or MinD protein is according to the invention also taken to mean the manipulation of the expression of the

plant-inherent, endogenous HMG-CoA reductase and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or 1-deoxy-D-xylose-5-phosphate synthase and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or isopentenyl-diphosphate Δ-isomerase and/or geranyl-diphosphate synthase and/or farnesyl-diphosphate synthase and/or geranylgeranyl-diphosphate synthase and/or phytoene synthase and/or phytoene desaturase and/or zeta-carotene desaturase and/or the plant-inherent crtISO protein and/or FtsZ protein and/or MinD protein.

This can be achieved, for example, by modifying the corresponding promoter DNA sequence. Such a modification which causes an elevated expression rate of the gene, can be achieved, for example, by deletion or insertion of DNA sequences.

In a preferred embodiment, the increase of the gene expression of a nucleic acid coding for an HMG-CoA reductase and/or the increase of the gene expression of a nucleic acid coding for an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or the increase of the gene expression of a nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate synthase and/or the increase of the gene expression of a nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or the increase of the gene expression of a nucleic acid coding for an isopentenyl-diphosphate Δ-isomerase and/or the increase of the gene expression of a nucleic acid coding for a geranyl-diphosphate synthase and/or the increase of the gene expression of a nucleic acid coding for a farnesyl-diphosphate synthase and/or the increase of the gene expression of a nucleic acid coding for a geranylgeranyl-diphosphate synthase and/or the increase of the gene expression of a nucleic acid coding for a phytoene synthase and/or the increase of the gene expression of a nucleic acid coding for a phytoene desaturase and/or the increase of the gene expression of a nucleic acid coding for a zeta-carotene desaturase and/or the increase of the gene expression of a nucleic acid coding for a crtISO protein and/or the increase of the gene expression of a nucleic acid coding for an FtsZ protein and/or the increase of the gene expression of a nucleic acid coding for a MinD protein is achieved by introducing at least one nucleic acid coding for an HMG-CoA reductase and/or by introducing at least one nucleic acid coding for an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or by introducing at least one nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate synthase and/or by introducing at least one nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or by introducing at least one nucleic acid coding for an

isopentenyl-diphosphate Δ-isomerase and/or by introducing at least one nucleic acid coding for a geranyl-diphosphate synthase and/or by introducing at least one nucleic acid coding for a farnesyl-diphosphate synthase and/or by introducing at least one nucleic acid coding for a geranylgeranyl-diphosphate synthase and/or by introducing at least one nucleic acid coding for a phytoene synthase and/or by introducing at least one nucleic acid coding for a phytoene desaturase and/or by introducing at least one nucleic acid coding for a zeta-carotene desaturase and/or by introducing at least one nucleic acid coding for a crtISO protein and/or by introducing at least one nucleic acid coding for an FtsZ protein and/or by introducing at least one nucleic acid coding for a MinD protein into the plant.

For this, in principle, use can be made of any HMG-CoA reductase gene or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase gene or 1-deoxy-D-xylose-5-phosphate synthase gene or 1-deoxy-D-xylose-5-phosphate reductoisomerase gene or isopentenyl-diphosphate Δ-isomerase gene or geranyl-diphosphate synthase gene or farnesyl-diphosphate synthase gene or geranylgeranyl-diphosphate synthase gene or phytoene synthase gene or phytoene desaturase gene or zeta-carotene desaturase gene or crtISO gene or FtsZ gene or MinD gene.

In genomic HMG-CoA reductase sequences or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase sequences or 1-deoxy-D-xylose-5-phosphate synthase sequences or 1-deoxy-D-xylose-5-phosphate reductoisomerase sequences or isopentenyl-diphosphate Δ-isomerase sequences or geranyl-diphosphate synthase sequences or farnesyl-diphosphate synthase sequences or geranylgeranyl-diphosphate synthase sequences or phytoene synthase sequences or phytoene desaturase sequences or zeta-carotene desaturase sequences or crtISO sequences or FtsZ sequences or MinD sequences from eukaryotic sources which comprise introns, in the event that the host plant is not able to, or cannot be given the ability to, express the corresponding proteins, preferably previously-processed nucleic acid sequences, such as the corresponding cDNAs, are to be used.

In the inventive preferred transgenic plants, therefore, in this preferred embodiment there is, compared with the wild type, at least one further HMG-CoA reductase gene and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase gene and/or 1-deoxy-D-xylose-5-phosphate synthase gene and/or 1-deoxy-D-xylose-5-phosphate

reductoisomerase gene and/or isopentenyl-diphosphate Δ-isomerase gene and/or geranyl-diphosphate synthase gene and/or farnesyl-diphosphate synthase gene and/or geranylgeranyl-diphosphate synthase gene and/or phytoene synthase gene and/or phytoene desaturase gene and/or zeta-carotene desaturase gene and/or crtISO gene and/or FtsZ gene and/or MinD gene.

In this preferred embodiment, the genetically modified plant has, for example, at least one exogenous nucleic acid coding for an HMG-CoA reductase or at least two endogenous nucleic acids coding for an HMG-CoA reductase and/or at least one exogenous nucleic acid coding for an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase or at least two endogenous nucleic acids coding for an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or at least one exogenous nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate synthase or at least two endogenous nucleic acids coding for a 1-deoxy-D-xylose-5-phosphate synthase and/or at least one exogenous nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate reductoisomerase or at least two endogenous nucleic acids coding for a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or at least one exogenous nucleic acid coding for an isopentenyl-diphosphate Δ-isomerase or at least two endogenous nucleic acids coding for an isopentenyl-diphosphate Δ-isomerase and/or at least one exogenous nucleic acid coding for a geranyl-diphosphate synthase or at least two endogenous nucleic acids coding for a geranyl-diphosphate synthase and/or at least one exogenous nucleic acid coding for a farnesyl-diphosphate synthase or at least two endogenous nucleic acids coding for a farnesyl-diphosphate synthase and/or at least one exogenous nucleic acid coding for a geranylgeranyl-diphosphate synthase or at least two endogenous nucleic acids coding for a geranylgeranyl-diphosphate synthase and/or at least one exogenous nucleic acid coding for a phytoene synthase or at least two endogenous nucleic acids coding for a phytoene synthase and/or at least one exogenous nucleic acid coding for a phytoene desaturase or at least two endogenous nucleic acids coding for a phytoene desaturase and/or at least one exogenous nucleic acid coding for a zeta-carotene desaturase or at least two endogenous nucleic acids coding for a zeta-carotene desaturase and/or at least one exogenous nucleic acid coding for a crtISO protein or at least two endogenous nucleic acids coding for a crtISO protein and/or at least one exogenous nucleic acid coding for an FtsZ protein or at least two endogenous nucleic acids coding for an FtsZ protein and/or at least one

exogenous nucleic acid coding for a MinD protein or at least two endogenous nucleic acids coding for a MinD protein.

Examples of HMG-CoA reductase genes are:

A nucleic acid coding for an HMG-CoA reductase from Arabidopsis thaliana, Accession NM_106299; (nucleic acid: SEQ ID NO: 111, protein: SEQ ID NO: 112),

and also further HMG-CoA reductase genes from other organisms having the following accession numbers:

P54961, P54870, P54868, P54869, O02734, P22791, P54873, P54871, P23228, P13704, P54872, Q01581, P17425, P54874, P54839, P14891, P34135, O64966, P29057, P48019, P48020, P12683, P43256, Q9XEL8, P34136, O64967, P29058, P48022, Q41437, P12684, Q00583, Q9XHL5, Q41438, Q9YAS4, O76819, O28538, Q9Y7D2, P54960, O51628, P48021, Q03163, P00347, P14773, Q12577, Q59468, P04035, O24594, P09610, Q58116, O26662, Q01237, Q01559, Q12649, O74164, O59469, P51639, Q10283, O08424, P20715, P13703, P13702, Q96UG4, Q8SQZ9, O15888, Q9TUM4, P93514, Q39628, P93081, P93080, Q944T9, Q40148, Q84MM0, Q84LS3, Q9Z9N4, Q9KLM0

Examples of (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes are:

A nucleic acid coding for an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase from Arabidopsis thaliana (lytB/ISPH), ACCESSION AY168881, (nucleic acid: SEQ ID NO: 113, protein: SEQ ID NO: 114),

and also further (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes from other organisms having the following accession numbers:

T04781, AF270978_1, NP_485028.1, NP_442089.1, NP_681832.1, ZP_00110421.1, ZP_00071594.1, ZP_00114706.1, ISPH_SYNY3, ZP_00114087.1, ZP_00104269.1, AF398145_1, AF398146_1, AAD55762.1, AF514843_1, NP_622970.1, NP_348471.1, NP_562001.1, NP_223698.1, NP_781941.1, ZP_00080042.1, NP_859669.1, NP_214191.1, ZP_00086191.1, ISPH_VIBCH, NP_230334.1, NP_742768.1, NP_302306.1, ISPH_MYCLE, NP_602581.1, ZP_00026966.1, NP_520563.1,

NP_253247.1, NP_282047.1, ZP_00038210.1, ZP_00064913.1, CAA61555.1, ZP_00125365.1, ISPH_ACICA, EAA24703.1, ZP_00013067.1, ZP_00029164.1, NP_790656.1, NP_217899.1, NP_641592.1, NP_636532.1, NP_719076.1, NP_660497.1, NP_422155.1, NP_715446.1, ZP_00090692.1, NP_759496.1, ISPH_BURPS, ZP_00129657.1, NP_215626.1, NP_335584.1, ZP_00135016.1, NP_789585.1, NP_787770.1, NP_769647.1, ZP_00043336.1, NP_242248.1, ZP_00008555.1, NP_246603.1, ZP_00030951.1, NP_670994.1, NP_404120.1, NP_540376.1, NP_733653.1, NP_697503.1, NP_840730.1, NP_274828.1, NP_796916.1, ZP_00123390.1, NP_824386.1, NP_737689.1, ZP_00021222.1, NP_757521.1, NP_390395.1, ZP_00133322.1, CAD76178.1, NP_600249.1, NP_454660.1, NP_712601.1, NP_385018.1, NP_751989.1

Examples of 1-deoxy-D-xylose-5-phosphate synthase genes are:

A nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate synthase from Lycopersicon esculentum, ACCESSION #AF143812 (nucleic acid: SEQ ID NO: 115 , protein: SEQ ID NO: 116),

and also further 1-deoxy-D-xylose-5-phosphate synthase genes from other organisms having the following accession numbers:

AF143812_1, DXS_CAPAN, CAD22530.1, AF182286_1, NP_193291.1, T52289, AAC49368.1, AAP14353.1, D71420, DXS_ORYSA, AF443590_1, BAB02345.1, CAA09804.2, NP_850620.1, CAD22155.2, AAM65798.1, NP_566686.1, CAD22531.1, AAC33513.1, CAC08458.1, AAG10432.1, T08140, AAP14354.1, AF428463_1, ZP_00010537.1, NP_769291.1, AAK59424.1, NP_107784.1, NP_697464.1, NP_540415.1, NP_196699.1, NP_384986.1, ZP_00096461.1, ZP_00013656.1, NP_353769.1, BAA83576.1, ZP_00005919.1, ZP_00006273.1, NP_420871.1, AAM48660.1, DXS_RHOCA, ZP_00045608.1, ZP_00031686.1, NP_841218.1, ZP_00022174.1, ZP_00086851.1, NP_742690.1, NP_520342.1, ZP_00082120.1, NP_790545.1, ZP_00125266.1, CAC17468.1, NP_252733.1, ZP_00092466.1, NP_439591.1, NP_414954.1, NP_752465.1, NP_622918.1, NP_286162.1, NP_836085.1, NP_706308.1, ZP_00081148.1, NP_797065.1, NP_213598.1, NP_245469.1, ZP_00075029.1, NP_455016.1, NP_230536.1, NP_459417.1, NP_274863.1, NP_283402.1, NP_759318.1, NP_406652.1, DXS_SYNLE,

DXS_SYNP7, NP_440409.1, ZP_00067331.1, ZP_00122853.1, NP_717142.1, ZP_00104889.1, NP_243645.1, NP_681412.1, DXS_SYNEL, NP_637787.1, DXS_CHLTE, ZP_00129863.1, NP_661241.1, DXS_XANCP, NP_470738.1, NP_484643.1, ZP_00108360.1, NP_833890.1, NP_846629.1, NP_658213.1, NP_642879.1, ZP_00039479.1, ZP_00060584.1, ZP_00041364.1, ZP_00117779.1, NP_299528.1

Examples of 1-deoxy-D-xylose-5-phosphate reductoisomerase genes are:

A nucleic acid coding for a 1-deoxy-D-xylose-5-phosphate reductoisomerase from Arabidopsis thaliana, ACCESSION #AF148852, (nucleic acid: SEQ ID NO: 137, protein: SEQ ID NO: 138),

and also further 1-deoxy-D-xylose-5-phosphate reductoisomerase genes from other organisms having the following accession numbers:

AF148852, AY084775, AY054682, AY050802, AY045634, AY081453, AY091405, AY098952, AJ242588, AB009053, AY202991, NP_201085.1, T52570, AF331705_1, BAB16915.1, AF367205_1, AF250235_1, CAC03581.1, CAD22156.1, AF182287_1, DXR_MENPI, ZP_00071219.1, NP_488391.1, ZP_00111307.1, DXR_SYNLE, AAP56260.1, NP_681831.1, NP_442113.1, ZP_00115071.1, ZP_00105106.1, ZP_00113484.1, NP_833540.1, NP_657789.1, NP_661031.1, DXR_BACHD, NP_833080.1, NP_845693.1, NP_562610.1, NP_623020.1, NP_810915.1, NP_243287.1, ZP_00118743.1, NP_464842.1, NP_470690.1, ZP_00082201.1, NP_781898.1, ZP_00123667.1, NP_348420.1, NP_604221.1, ZP_00053349.1, ZP_00064941.1, NP_246927.1, NP_389537.1, ZP_00102576.1, NP_519531.1, AF124757_19, DXR_ZYMMO, NP_713472.1, NP_459225.1, NP_454827.1, ZP_00045738.1, NP_743754.1, DXR_PSEPK, ZP_00130352.1, NP_702530.1, NP_841744.1, NP_438967.1, AF514841_1, NP_706118.1, ZP_00125845.1, NP_404661.1, NP_285867.1, NP_240064.1, NP_414715.1, ZP_00094058.1, NP_791365.1, ZP_00012448.1, ZP_00015132.1, ZP_00091545.1, NP_629822.1, NP_771495.1, NP_798691.1, NP_231885.1, NP_252340.1, ZP_00022353.1, NP_355549.1, NP_420724.1, ZP_00085169.1, EAA17616.1, NP_273242.1, NP_219574.1, NP_387094.1, NP_296721.1, ZP_00004209.1, NP_823739.1, NP_282934.1, BAA77848.1, NP_660577.1, NP_760741.1, NP_641750.1,

NP_636741.1, NP_829309.1, NP_298338.1, NP_444964.1, NP_717246.1, NP_224545.1, ZP_00038451.1, DXR_KITGR, NP_778563.1.

Examples of isopentenyl-diphosphate Δ-isomerase genes are:

A nucleic acid coding for an isopentenyl-diphosphate Δ-isomerase from Adonis palaestina clone ApIPI28, (ipiAa1), ACCESSION #AF188060, published by Cunningham,F.X. Jr. and Gantt,E.: Identification of multi-gene families encoding isopentenyl diphosphate isomerase in plants by heterologous complementation in Escherichia coli, Plant Cell Physiol. 41 (1), 119-123 (2000) (nucleic acid: SEQ ID NO: 117, protein: SEQ ID NO: 118),

and also further isopentenyl-diphosphate Δ-isomerase genes from other organisms having the following accession numbers:

Q38929, O48964, Q39472, Q13907, O35586, P58044, O42641, O35760, Q10132, P15496, Q9YB30, Q8YNH4, Q42553, O27997, P50740, O51627, O48965, Q8KFR5, Q39471, Q39664, Q9RVE2, Q01335, Q9HHE4, Q9BXS1, Q9KWF6, Q9CIF5, Q88WB6, Q92BX2, Q8Y7A5, Q8TT35 Q9KK75, Q8NN99, Q8XD58, Q8FE75, Q46822, Q9HP40, P72002, P26173, Q9Z5D3, Q8Z3X9, Q8ZM82, Q9X7Q6, O13504, Q9HFW8, Q8NJL9, Q9UUQ1, Q9NH02, Q9M6K9, Q9M6K5, Q9FXR6, O81691, Q9S7C4, Q8S3L8, Q9M592, Q9M6K3, Q9M6K7, Q9FV48, Q9LLB6, Q9AVJ1, Q9AVG8, Q9M6K6, Q9AVJ5, Q9M6K2, Q9AYS5, Q9M6K8, Q9AVG7, Q8S3L7, Q8W250, Q94IE1, Q9AVI8, Q9AYS6, Q9SAY0, Q9M6K4, Q8GVZ0, Q84RZ8, Q8KZ12, Q8KZ66, Q8FND7, Q88QC9, Q8BFZ6, BAC26382, CAD94476.

Examples of geranyl-diphosphate synthase genes are:

A nucleic acid coding for a geranyl-diphosphate synthase from Arabidopsis thaliana, ACCESSION #Y17376, Bouvier,F., Suire,C., d'Harlingue,A., Backhaus,R.A. and Camara,B.: Molecular cloning of geranyl diphosphate synthase and compartmentation of monoterpene synthesis in plant cells, Plant J. 24 (2), 241-252 (2000) (nucleic acid: SEQ ID NO: 119, protein: SEQ ID NO: 120),

and also further geranyl-diphosphate synthase genes from other organisms having the following accession numbers:

Q9FT89, Q8LKJ2, Q9FSW8, Q8LKJ3, Q9SBR3, Q9SBR4, Q9FET8, Q8LKJ1, Q84LG1, Q9JK86

Examples of farnesyl-diphosphate synthase genes are:

A nucleic acid coding for a farnesyl-diphosphate synthase from Arabidopsis thaliana (FPS1), ACCESSION #U80605, published by Cunillera,N., Arro,M., Delourme,D., Karst,F., Boronat,A. and Ferrer,A.: Arabidopsis thaliana comprises two differentially expressed farnesyl-diphosphate synthase genes, J. Biol. Chem. 271 (13), 7774-7780 (1996), (nucleic acid: SEQ ID NO: 121, protein: SEQ ID NO: 122),

and also further farnesyl-diphosphate synthase genes from other organisms having the following accession numbers:

P53799, P37268, Q02769, Q09152, P49351, O24241, Q43315, P49352, O24242, P49350, P08836, P14324, P49349, P08524, O66952, Q08291, P54383, Q45220, P57537, Q8K9A0, P22939, P45204, O66126, P55539, Q9SWH9, Q9AVI7, Q9FRX2, Q9AYS7, Q94IE8, Q9FXR9, Q9ZWF6, Q9FXR8, Q9AR37, O50009,Q94IE9,Q8RVK7, Q8RVQ7, O04882, Q93RA8, Q93RB0, Q93RB4, Q93RB5,Q93RB3, Q93RB1, Q93RB2, Q920E5.

Examples of geranylgeranyl-diphosphate synthase genes are:

A nucleic acid coding for a geranylgeranyl-diphosphate synthase from Sinapis alba, ACCESSION #X98795, published by Bonk,M., Hoffmann,B., Von Lintig,J., Schledz,M., Al-Babili,S., Hobeika,E., Kleinig,H. and Beyer,P.: Chloroplast import of four carotenoid biosynthetic enzymes in vitro reveals differential fates prior to membrane binding and oligomeric assembly, Eur. J. Biochem. 247 (3), 942-950 (1997), (nucleic acid: SEQ ID NO: 123, protein: SEQ ID NO: 124),

and also further geranylgeranyl-diphosphate synthase genes from other organisms having the following accession numbers:

P22873, P34802 ,P56966, P80042, Q42698, Q92236, O95749, Q9WTN0, Q50727, P24322, P39464, Q9FXR3, Q9AYN2, Q9FXR2, Q9AVG6, Q9FRW4, Q9SXZ5,

Q9AVJ7, Q9AYN1, Q9AVJ4, Q9FXR7, Q8LSC5, Q9AVJ6, Q8LSC4, Q9AVJ3, Q9SSU0, Q9SXZ6, Q9SST9, Q9AVJ0, Q9AVI9, Q9FRW3, Q9FXR5, Q94IF0, Q9FRX1, Q9K567, Q93RA9, Q93QX8, CAD95619, EAA31459

Examples of phytoene synthase genes are:

A nucleic acid coding for a phytoene synthase from Erwinia uredovora, ACCESSION # D90087, published by Misawa,N., Nakagawa,M., Kobayashi,K., Yamano,S., Izawa,Y.,Nakamura,K. and Harashima,K.: Elucidation of the Erwinia uredovora carotenoid biosynthetic pathway by functional analysis of gene products expressed in Escherichia coli; J. Bacteriol. 172 (12), 6704-6712 (1990), (nucleic acid: SEQ ID NO: 125, protein: SEQ ID NO: 126),

and also further phytoene synthase genes from other organisms having the following accession numbers:

CAB39693, BAC69364, AAF10440, CAA45350, BAA20384, AAM72615, BAC09112, CAA48922, P_001091, CAB84588, AAF41518, CAA48155, AAD38051, AAF33237, AAG10427, AAA34187, BAB73532, CAC19567, AAM62787, CAA55391, AAB65697, AAM45379, CAC27383, AAA32836, AAK07735, BAA84763, P_000205, AAB60314, P_001163, P_000718, AAB71428, AAA34153, AAK07734, CAA42969, CAD76176, CAA68575, P_000130, P_001142, CAA47625, CAA85775, BAC14416, CAA79957, BAC76563, P_000242, P_000551, AAL02001, AAK15621, CAB94795, AAA91951, P_000448

Examples of phytoene desaturase genes are:

A nucleic acid coding for a phytoene desaturase from Erwinia uredovora, ACCESSION # D90087, published by Misawa,N., Nakagawa,M., Kobayashi,K., Yamano,S., Izawa,Y., Nakamura,K. and Harashima,K.: Elucidation of the Erwinia uredovora carotenoid biosynthetic pathway by functional analysis of gene products expressed in Escherichia coli; J. Bacteriol. 172 (12), 6704-6712 (1990), (nucleic acid: SEQ ID NO: 127, protein: SEQ ID NO: 128),

and also further phytoene desaturase genes from other organisms having the following accession numbers:

AAL15300, A39597, CAA42573, AAK51545, BAB08179, CAA48195, BAB82461, AAK92625, CAA55392, AAG10426, AAD02489, AAO24235, AAC12846, AAA99519, AAL38046, CAA60479, CAA75094, ZP_001041, ZP_001163, CAA39004, CAA44452, ZP_001142, ZP_000718, BAB82462, AAM45380, CAB56040, ZP_001091, BAC09113, AAP79175, AAL80005, AAM72642, AAM72043, ZP_000745, ZP_001141, BAC07889, CAD55814, ZP_001041, CAD27442, CAE00192, ZP_001163, ZP_000197, BAA18400, AAG10425, ZP_001119, AAF13698, 2121278A, AAB35386, AAD02462, BAB68552, CAC85667, AAK51557, CAA12062, AAG51402, AAM63349, AAF85796, BAB74081, AAA91161, CAB56041, AAC48983, AAG14399, CAB65434, BAB73487, ZP_001117, ZP_000448, CAB39695, CAD76175, BAC69363, BAA17934, ZP_000171, AAF65586, ZP_000748, BAC07074, ZP_001133, CAA64853, BAB74484, ZP_001156, AAF23289, AAG28703, AAP09348, AAM71569, BAB69140, ZP_000130, AAF41516, AAG18866, CAD95940, NP_656310, AAG10645, ZP_000276, ZP_000192, ZP_000186, AAM94364, EAA31371, ZP_000612, BAC75676, AAF65582

Examples of zeta-carotene desaturase genes are:

A nucleic acid coding for a zeta-carotene desaturase from Narcissus pseudonarcissus, ACCESSION #AJ224683, published by Al-Babili,S., Oelschlegel,J. and Beyer,P.: A cDNA encoding for beta carotene desaturase (Accession No.AJ224683) from Narcissus pseudonarcissus L.. (PGR98-103), Plant Physiol. 117, 719-719 (1998), (nucleic acid: SEQ ID NO: 129, protein: SEQ ID NO: 130),

and also further zeta-carotene desaturase genes from other organisms having the following accession numbers:

Q9R6X4, Q38893, Q9SMJ3, Q9SE20, Q9ZTP4, O49901, P74306, Q9FV46, Q9RCT2, ZDS_NARPS, BAB68552.1, CAC85667.1, AF372617_1, ZDS_TARER, CAD55814.1, CAD27442.1, 2121278A, ZDS_CAPAN, ZDS_LYCES, NP_187138.1, AAM63349.1, ZDS_ARATH, AAA91161.1, ZDS_MAIZE, AAG14399.1, NP_441720.1, NP_486422.1, ZP_00111920.1, CAB56041.1, ZP_00074512.1, ZP_00116357.1, NP_681127.1, ZP_00114185.1, ZP_00104126.1, CAB65434.1, NP_662300.1

Examples of crtISO genes are:

A nucleic acid coding for a crtISO from Lycopersicon esculentum; ACCESSION #AF416727, published by Isaacson,T., Ronen,G., Zamir,D. and Hirschberg,J.: Cloning of tangerine from tomato reveals a carotenoid isomerase essential for the production of beta-carotene and xanthophylls in plants; Plant Cell 14 (2), 333-342 (2002), (nucleic acid: SEQ ID NO: 131, protein: SEQ ID NO: 132),

and also further crtISO genes from other organisms having the following accession numbers:

AAM53952

Examples of FtsZ genes are:

A nucleic acid coding for an FtsZ from Tagetes erecta, ACCESSION #AF251346, published by Moehs,C.P., Tian,L., Osteryoung,K.W. and Dellapenna,D.: Analysis of carotenoid biosynthetic gene expression during marigold petal development; Plant Mol. Biol. 45 (3), 281-293 (2001), (nucleic acid: SEQ ID NO: 133, protein: SEQ ID NO: 134),

and also further FtsZ genes from other organisms having the following accession numbers:

CAB89286.1, AF205858_1, NP_200339.1, CAB89287.1, CAB41987.1, AAA82068.1, T06774,AF383876_1, BAC57986.1, CAD22047.1, BAB91150.1, ZP_00072546.1, NP_440816.1, T51092, NP_683172.1, BAA85116.1, NP_487898.1, JC4289, BAA82871.1, NP_781763.1, BAC57987.1, ZP_00111461.1, T51088, NP_190843.1, ZP_00060035.1, NP_846285.1, AAL07180.1, NP_243424.1, NP_833626.1, AAN04561.1, AAN04557.1, CAD22048.1, T51089, NP_692394.1, NP_623237.1, NP_565839.1, T51090, CAA07676.1, NP_113397.1, T51087, CAC44257.1, E84778, ZP_00105267.1, BAA82091.1, ZP_00112790.1, BAA96782.1, NP_348319.1, NP_471472.1, ZP_00115870.1, NP_465556.1, NP_389412.1, BAA82090.1, NP_562681.1, AAM22891.1, NP_371710.1, NP_764416.1, CAB95028.1, FTSZ_STRGR, AF120117_1, NP_827300.1, JE0282, NP_626341.1, AAC45639.1, NP_785689.1, NP_336679.1, NP_738660.1, ZP_00057764.1, AAC32265.1, NP_814733.1, FTSZ_MYCKA, NP_216666.1, CAA75616.1, NP_301700.1, NP_601357.1, ZP_00046269.1, CAA70158.1, ZP_00037834.1, NP_268026.1, FTSZ_ENTHR, NP_787643.1, NP_346105.1, AAC32264.1, JC5548, AAC95440.1,

NP_710793.1, NP_687509.1, NP_269594.1, AAC32266.1, NP_720988.1, NP_657875.1, ZP_00094865.1, ZP_00080499.1, ZP_00043589.1, JC7087, NP_660559.1, AAC46069.1, AF179611_14, AAC44223.1, NP_404201.1.

Examples of MinD genes are:

A nucleic acid coding for a MinD from Tagetes erecta, ACCESSION #AF251019, published by Moehs,C.P., Tian,L., Osteryoung,K.W. and Dellapenna,D.: Analysis of carotenoid biosynthetic gene expression during marigold petal development; Plant Mol. Biol. 45 (3), 281-293 (2001), (nucleic acid: SEQ ID NO: 135, protein: SEQ ID NO: 136),

and also further MinD genes having the following accession numbers:

NP_197790.1, BAA90628.1, NP_038435.1, NP_045875.1, AAN33031.1, NP_050910.1, CAB53105.1, NP_050687.1, NP_682807.1, NP_487496.1, ZP_00111708.1, ZP_00071109.1, NP_442592.1, NP_603083.1, NP_782631.1, ZP_00097367.1, ZP_00104319.1, NP_294476.1, NP_622555.1, NP_563054.1, NP_347881.1, ZP_00113908.1, NP_834154.1, NP_658480.1, ZP_00059858.1, NP_470915.1, NP_243893.1, NP_465069.1, ZP_00116155.1, NP_390677.1, NP_692970.1, NP_298610.1, NP_207129.1, ZP_00038874.1, NP_778791.1, NP_223033.1, NP_641561.1, NP_636499.1, ZP_00088714.1, NP_213595.1, NP_743889.1, NP_231594.1, ZP_00085067.1, NP_797252.1, ZP_00136593.1, NP_251934.1, NP_405629.1, NP_759144.1, ZP_00102939.1, NP_793645.1, NP_699517.1, NP_460771.1, NP_860754.1, NP_456322.1, NP_718163.1, NP_229666.1, NP_357356.1, NP_541904.1, NP_287414.1, NP_660660.1, ZP_00128273.1, NP_103411.1, NP_785789.1, NP_715361.1, AF149810_1, NP_841854.1, NP_437893.1, ZP_00022726.1, EAA24844.1, ZP_00029547.1, NP_521484.1, NP_240148.1, NP_770852.1, AF345908_2, NP_777923.1, ZP_00048879.1, NP_579340.1, NP_143455.1, NP_126254.1, NP_142573.1, NP_613505.1, NP_127112.1, NP_712786.1, NP_578214.1, NP_069530.1, NP_247526.1, AAA85593.1, NP_212403.1, NP_782258.1, ZP_00058694.1, NP_247137.1, NP_219149.1, NP_276946.1, NP_614522.1, ZP_00019288.1, CAD78330.1

Preferably, in the above-described preferred embodiment, as HMG-CoA reductase genes, use is made of nucleic acids which code for proteins comprising the amino acid

sequence SEQ ID NO: 112 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 112, and which have the enzymatic property of an HMG-CoA reductase.

Further examples of HMG-CoA reductases and HMG-CoA reductase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 112.

Further examples of HMG-CoA reductases and HMG-CoA reductase genes may furthermore readily be found, for example, starting from the sequence SEQ ID NO: 111 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the HMG-CoA reductase activity, nucleic acids which code for proteins comprising the amino acid sequence of the HMG-CoA reductase of the sequence SEQ ID NO: 112 are introduced into organisms.

Suitable nucleic acid sequences are, for example, obtainable by back-translation of the polypeptide sequence according to the genetic code.

Preferably, for this, those codons are used which are used frequently in accordance with the plant-specific codon usage. The codon usage may be readily determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 111 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 114 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which

proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 114 and which have the enzymatic property of an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase.

Further examples of (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductases and (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes may be readily found, for example, from various organisms whose genomic sequence is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 114.

Further examples of (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductases and (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes may furthermore readily be found, for example, starting from the sequence SEQ ID NO: 113 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, nucleic acids are introduced into organisms which code for proteins comprising the amino acid sequence of the (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase of the sequence SEQ ID NO: 114.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 113 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as 1-deoxy-D-xylose-5-phosphate synthase genes, use is made of nucleic acids which code for proteins

comprising the amino acid sequence SEQ ID NO: 116 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 116 and which have the enzymatic property of a 1-deoxy-D-xylose-5-phosphate synthase.

Further examples of 1-deoxy-D-xylose-5-phosphate synthases and 1-deoxy-D-xylose-5-phosphate synthase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 116.

Further examples of 1-deoxy-D-xylose-5-phosphate synthases and 1-deoxy-D-xylose-5-phosphate synthase genes may in addition readily be found, for example, starting from the sequence SEQ ID NO: 115 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the 1-deoxy-D-xylose-5-phosphate synthase activity, nucleic acids which code for proteins comprising the amino acid sequence of the 1-deoxy-D-xylose-5-phosphate synthase of the sequence SEQ ID NO: 116 are introduced into organisms.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, those codons are used which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 115 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as 1-deoxy-D-xylose-5-phosphate reductoisomerase genes, use is made of nucleic acids which code for

proteins comprising the amino acid sequence SEQ ID NO: 138 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 138 and which have the enzymatic property of a 1-deoxy-D-xylose-5-phosphate reductoisomerase.

Further examples of 1-deoxy-D-xylose-5-phosphate reductoisomerases and 1-deoxy-D-xylose-5-phosphate reductoisomerase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 138.

Further examples of 1-deoxy-D-xylose-5-phosphate reductoisomerases and 1-deoxy-D-xylose-5-phosphate reductoisomerase genes may in addition readily be found, for example, starting from the sequence SEQ ID NO: 137 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the 1-deoxy-D-xylose-5-phosphate reductoisomerase of the sequence SEQ ID NO: 138.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 137 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as isopentenyl D-isomerase genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 118, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 118 and which have the enzymatic property of an isopentenyl D-isomerase.

Further examples of isopentenyl D-isomerases and isopentenyl D-isomerase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 118.

Further examples of isopentenyl D-isomerases and isopentenyl D-isomerase genes may in addition readily be found, for example, starting from the sequence SEQ ID NO: 117 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the isopentenyl D-isomerase activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the isopentenyl D-isomerase of the sequence SEQ ID NO: 118.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 117 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as geranyl-diphosphate synthase genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 120 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 120 and which have the enzymatic property of a geranyl-diphosphate synthase.

Further examples of geranyl-diphosphate synthases and geranyl-diphosphate synthase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 120.

Further examples of geranyl-diphosphate synthases and geranyl-diphosphate synthase genes may in addition readily be found, for example, starting from the sequence SEQ ID NO: 119 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the geranyl-diphosphate synthase activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the geranyl-diphosphate synthase of the sequence SEQ ID NO: 120.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 119 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as farnesyl-diphosphate synthase genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 122 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 122 and which have the enzymatic property of a farnesyl-diphosphate synthase.

Further examples of farnesyl-diphosphate synthases and farnesyl-diphosphate synthase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 122.

Further examples of farnesyl-diphosphate synthases and farnesyl-diphosphate synthase genes may, in addition, readily be found, for example, starting from the sequence SEQ ID NO: 121 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the farnesyl-diphosphate synthase activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the farnesyl-diphosphate synthase of the sequence SEQ ID NO: 122.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 121 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as geranylgeranyl-diphosphate synthase genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 124 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 124 and which have the enzymatic property of a geranylgeranyl-diphosphate synthase.

Further examples of geranylgeranyl-diphosphate synthases and geranylgeranyl-diphosphate synthase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 124.

Further examples of geranylgeranyl-diphosphate synthases and geranylgeranyl-diphosphate synthase genes may in addition readily be found, for example, starting from the sequence SEQ ID NO: 123 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the geranylgeranyl-diphosphate synthase activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the geranylgeranyl-diphosphate synthase of the sequence SEQ ID NO: 124.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 123 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as phytoene synthase genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 126 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 126 and which have the enzymatic property of a phytoene synthase.

Further examples of phytoene synthases and phytoene synthase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 126.

Further examples of phytoene synthases and phytoene synthase genes may in addition readily be found, for example, starting from the sequence SEQ ID NO: 125 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the phytoene synthase activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the phytoene synthase of the sequence SEQ ID NO: 126.

Suitable nucleic acid sequences are, for example, obtainable by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are frequently used in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 125 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as phytoene desaturase genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 128 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 128, and which have the enzymatic property of a phytoene desaturase.

Further examples of phytoene desaturases and phytoene desaturase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 128.

Further examples of phytoene desaturases and phytoene desaturase genes may in addition readily be found, for example, starting from the sequence SEQ ID NO: 127 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the phytoene desaturase activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the phytoene desaturase of the sequence SEQ ID NO: 128.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are frequently used in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 127 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as zeta-carotene desaturase genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 130 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 130, and which have the enzymatic property of a zeta-carotene desaturase.

Further examples of zeta-carotene desaturases and zeta-carotene desaturase genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 130.

Further examples of zeta-carotene desaturases and zeta-carotene desaturase genes may in addition readily be found, for example, starting from the sequence SEQ ID NO: 129 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the zeta-carotene desaturase activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the zeta-carotene desaturase of the sequence SEQ ID NO: 130.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 129 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as CrtIso genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 132 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 132, and which have the enzymatic property of a CrtIso.

Further examples of CrtIsos and CrtIso genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 132.

Further examples of CrtIsos and CrtIso genes may, in addition, readily be found, for example, starting from the sequence SEQ ID NO: 131 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the CrtIso activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the CrtIso of the sequence SEQ ID NO: 132.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 131 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as FtsZ genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 134 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 134, and which have the enzymatic property of an FtsZ.

Further examples of FtsZs and FtsZ genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 134.

Further examples of FtsZs and FtsZ genes may, in addition, readily be found, for example, starting from the sequence SEQ ID NO: 133 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the FtsZ activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the FtsZ of the sequence SEQ ID NO: 134.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 133 is introduced into the organism.

Preferably, in the above-described preferred embodiment, as MinD genes, use is made of nucleic acids which code for proteins comprising the amino acid sequence SEQ ID NO: 136 or a sequence derived from this sequence by substitution, insertion or deletion

of amino acids, which proteins have an identity of at least 30%, preferably at least 50%, more preferably at least 70%, still more preferably at least 90%, most preferably at least 95%, at the amino acid level, with the sequence SEQ ID NO: 136 and which have the enzymatic property of a MinD.

Further examples of MinDs and MinD genes may readily be found, for example, from various organisms, the genomic sequence of which is known, as described above, by comparisons of homology of the amino acid sequences or of the corresponding back-translated nucleic acid sequences from databases with the SEQ ID NO: 136.

Further examples of MinDs and MinD genes may, in addition, readily be found, for example, starting from the sequence SEQ ID NO: 135 from various organisms, the genomic sequence of which is not known, as described above, by hybridization and PCR techniques in a manner known per se.

In a further particularly preferred embodiment, to increase the MinD activity, nucleic acids are introduced into organisms, which nucleic acids code for proteins comprising the amino acid sequence of the MinD of the sequence SEQ ID NO: 136.

Suitable nucleic acid sequences are obtainable, for example, by back-translation of the polypeptide sequence in accordance with the genetic code.

Preferably, for this, use is made of those codons which are used frequently in accordance with the plant-specific codon usage. The codon usage may readily be determined on the basis of computer evaluations of other known genes of the relevant organisms.

In a particularly preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 135 is introduced into the organism.

All of the abovementioned HMG-CoA reductase genes, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes, 1-deoxy-D-xylose-5-phosphate synthase genes, 1-deoxy-D-xylose-5-phosphate reductoisomerase genes, isopentenyl-diphosphate Δ-isomerase genes, geranyl-diphosphate synthase genes, farnesyl-diphosphate synthase genes, geranylgeranyl-diphosphate synthase genes, phytoene synthase genes, phytoene desaturase genes, zeta-carotene desaturase genes, crtISO genes, FtsZ

genes or MinD genes, furthermore, can be prepared in a manner known per se by chemical synthesis from the nucleotide building blocks, for example by fragment condensation of individual overlapping complementary nucleic acid building blocks of the double helix. The chemical synthesis of oligonucleotides can be performed, for example, in a known manner by the phosphoamidite method (Voet, Voet, 2nd edition, Wiley Press New York, pages 896-897). The attachment of synthetic oligonucleotides and filling-in of gaps using the Klenow fragment of the DNA polymerase and ligation reactions and also general cloning methods are described in Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press.

In a further preferred embodiment of the method, the plants additionally have a reduced endogenous β-hydroxylase activity compared with the wild type.

A reduced activity is, as mentioned above, preferably taken to mean the partial or essentially complete suppression or blocking, based on differing mechanisms of cell biology, of the functionality of an enzyme in a plant cell, plant or part derived therefrom, tissue, organ, cells or seeds.

The reduction of an activity in plants compared with the wild type can be achieved, for example, by reducing the amount of protein, or the amount of mRNA in the plant. Accordingly, an activity reduced compared with the wild type can be determined directly, or via the determination of the amount of protein or amount of mRNA of the inventive plant compared with the wild type.

A reduction of an activity comprises a quantitative decrease of a protein up to an essentially complete absence of the protein (that is to say absence of detectability of the corresponding activity or absence of immunological detectability of the corresponding protein).

Endogenous β-hydroxylase activity is taken to mean the enzyme activity of the endogenous plant-inherent β-hydroxylase.

An endogenous β-hydroxylase is taken to mean an endogenous plant-inherent hydroxylase as described above. If, for example, Tagetes erecta is the target plant to

be genetically modified, the endogenous β-hydroxylase is taken to mean the β-hydroxylase of Tagetes erecta.

An endogenous β-hydroxylase is therefore taken to mean, in particular, a plant-inherent protein which has the enzymatic activity to convert β-carotene to zeaxanthin.

Accordingly, endogenous β-hydroxylase activity is taken to mean the amount of β-carotene converted or amount of zeaxanthin formed by the protein endogenous β-hydroxylase in a defined time.

In the case of a reduced endogenous β-hydroxylase activity compared with the wild type, the amount of β-carotene converted or the amount of zeaxanthin formed by the protein endogenous β-hydroxylase is reduced in a defined time compared with the wild type.

Preferably, this reduction of the endogenous β-hydroxylase activity is at least 5%, further preferably at least 20%, further preferably at least 50%, further preferably 100%. Particularly preferably, the endogenous β-hydroxylase activity is completely switched off.

It has surprisingly been found that in plants which by a majority produce carotenoids of the α-carotene pathway, for example lutein, for example plants of the genus Tagetes, it is advantageous to reduce the activity of the endogenous β-hydroxylase and if appropriate to increase the activity of a heterologous hydroxylase. Particularly preferably, use is made of hydroxylases or functional equivalents thereof which originate from plants which produce by a majority carotenoids of the β-carotene pathway, for example the above-described β-hydroxylase from tomato (nucleic acid: SEQ ID No. 107, protein: SEQ ID No. 108).

The endogenous β-hydroxylase activity is determined as described above in a similar manner to determination of hydroxylase activity.

Preferably, the endogenous β–hydroxylase activity in plants is reduced by at least one of the following methods:

a) introducing at least one double-stranded endogenous β–hydroxylase ribonucleic acid sequence, hereinafter also termed endogenous β–hydroxylase-dsRNA, or an expression cassette ensuring expression thereof, or expression cassettes.

Those methods are comprised in which the endogenous β–hydroxylase-dsRNA is directed against an endogenous β–hydroxylase gene (that is to say genomic DNA sequences such as the promoter sequence) or an endogenous β–hydroxylase transcript (that is to say mRNA sequences),

b) introducing at least one endogenous β–hydroxylase antisense ribonucleic acid sequence, hereinafter also termed endogenous β–hydroxylase-antisense RNA, or an expression cassette ensuring expression thereof. Those methods are comprised in which the endogenous β–hydroxylase-antisense RNA is directed against an endogenous β–hydroxylase gene (that is to say genomic DNA sequences) or an endogenous β–hydroxylase gene transcript (that is to say RNA sequences). α-Anomeric nucleic acid sequences are also comprised

c) introducing at least one endogenous β–hydroxylase-antisense RNA combined with a ribozyme or an expression cassette ensuring expression thereof

d) introducing at least one endogenous β–hydroxylase sense ribonucleic acid sequence, hereinafter also termed endogenous β–hydroxylase-sense RNA, for inducing a cosuppression or an expression cassette ensuring expression thereof

e) introducing at least one DNA- or protein-binding factor against an endogenous β–hydroxylase gene, β–hydroxylase RNA or β–hydroxylase protein or an expression cassette ensuring expression thereof

f) introducing at least one viral nucleic acid sequence, or an expression cassette ensuring expression thereof, causing breakdown of the endogenous β–hydroxylase RNA

g) introducing at least one construct for generating a loss of function, for example the generation of stop codons or a shift in the reading frame, in an endogenous β–hydroxylase gene, for example by generating an insertion, deletion, inversion

or mutation in an endogenous β–hydroxylase gene. Preferably, knockout mutants can be generated by means of targeted insertion into said endogenous β–hydroxylase gene by homologous recombination or introduction of sequence-specific nucleases against endogenous β–hydroxylase gene sequences.

Those skilled in the art know that other methods can also be used in the context of the present invention for reducing an endogenous β–hydroxylase or activity thereof, or function. For example, introducing a dominant-negative variant of an endogenous β–hydroxylase or an expression cassette ensuring expression thereof can also be advantageous. Each individual one of these methods can cause a reduction of the amount of protein, amount of mRNA and/or activity of an endogenous β–hydroxylase. Combined use is also conceivable. Further methods are known to those skilled in the art and can comprise the inhibition or suppression of processing of the endogenous β–hydroxylase, of the transport of the zeaxanthin epoxidase and/or endogenous β–hydroxylase or mRNA thereof, inhibition of ribosome attachment, inhibition of RNA splicing, induction of an endogenous β–hydroxylase-RNA-degrading enzyme and/or inhibition of the elongation or termination of translation.

The individual preferred methods may be described hereinafter by exemplary embodiments:

a) introducing a double-stranded endogenous β–hydroxylase ribonucleic acid sequence (endogenous β–hydroxylase-dsRNA)

The method of gene regulation by means of double-stranded RNA has been described extensively above for reducing the ε-cyclase activity. In a similar manner, this method may be carried out for reducing the endogenous β–hydroxylase activity.

A double-stranded endogenous β–hydroxylase ribonucleic acid sequence or else endogenous β–hydroxylase-dsRNA is preferably taken to mean an RNA molecule which has a region having double-stranded structure and, in this region, comprises a nucleic acid sequence which

a) is identical to at least a part of the plant-inherent endogenous β–hydroxylase transcript and/or

b) is identical to at least a part of the plant-inherent endogenous β–hydroxylase promoter sequence.

In the inventive method, for the reduction of the endogenous β–hydroxylase activity, preferably an RNA is introduced into the plant, which RNA has a region having double-stranded structure and, in this region, comprises a nucleic acid sequence which

a) is identical to at least a part of the plant-inherent endogenous β–hydroxylase transcript and/or

b) is identical to at least a part of the plant-inherent endogenous β–hydroxylase promoter sequence.

The term "endogenous β–hydroxylase transcript" is taken to mean the transcribed part of an endogenous β–hydroxylase gene which, in addition to the sequence coding for the endogenous β–hydroxylase, also comprises, for example, non-coding sequences, for example also UTRs.

An RNA which "is identical to at least a part of the plant-inherent endogenous β–hydroxylase promoter sequence" is preferably taken to mean the fact that the RNA sequence is identical to at least a part of the theoretical transcript of the endogenous β–hydroxylase promoter sequence, that is to say the corresponding RNA sequence.

"A part" of the plant-inherent endogenous β–hydroxylase transcript or of the plant-inherent endogenous β–hydroxylase promoter sequence is taken to mean partial sequences which can range from a few base pairs up to complete sequences of the transcript or of the promoter sequence. The optimum length of the partial sequences can readily be determined by those skilled in the art by routine experiments.

Generally, the length of the partial sequences is at least 10 bases and at most 2 kb, preferably at least 25 bases and at most 1.5 kb, particularly preferably at least 50 bases and at most 600 bases, very particularly preferably at least 100 bases and at most 500, most preferably at least 200 bases or at least 300 bases and at most 400 bases.

Preferably, the partial sequences are sought out in such a manner that a specificity as high as possible is achieved and activities of other enzymes, the reduction of which is not desired, are not reduced. It is therefore advantageous for the partial sequences of the endogenous β–hydroxylase-dsRNA to select parts of the endogenous β–hydroxylase transcript and/or partial sequences of the endogenous β–hydroxylase promoter sequences which do not occur in other activities.

In a particularly preferred embodiment, therefore, the endogenous β–hydroxylase-dsRNA comprises a sequence which is identical to a part of the plant-inherent endogenous β–hydroxylase transcript and comprises the 5' end or the 3' end of the plant-inherent nucleic acid coding for an endogenous β–hydroxylase. In particular, non-translated regions in the 5' or 3' of the transcript are suitable for producing selective double-stranded structures.

The invention further relates to double-stranded RNA molecules (dsRNA molecules) which, on introduction into a plant organism (or a cell, tissue, organ or propagated material derived therefrom), cause the reduction of an endogenous β–hydroxylase.

The invention further relates to a double-stranded RNA molecule for reducing the expression of an endogenous β–hydroxylase (endogenous β–hydroxylase-dsRNA), preferably comprising

a) a "sense" RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of a "sense" RNA-endogenous β–hydroxylase transcript, and

b) an "antisense" RNA strand which is essentially, preferably completely, complementary to the RNA "sense" strand under a).

For the transformation of the plant with an endogenous β–hydroxylase-dsRNA, preferably a nucleic acid construct is used which is introduced into the plant and which is transcribed in the plant into the endogenous β–hydroxylase-dsRNA.

The present invention also further relates to a nucleic acid construct which can be transcribed into

a) a "sense" RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of the "sense" RNA endogenous β–hydroxylase transcript, and

b) an "antisense" RNA strand which is essentially, preferably completely, complementary to the RNA sense strand under a).

These nucleic acid constructs are also termed hereinafter expression cassettes or expression vectors.

With respect to the dsRNA molecules, the endogenous β–hydroxylase nucleic acid sequence, or the corresponding transcript, is preferably taken to mean the sequence according to SEQ ID NO: 139 or a part of same.

"Essentially identical" means that the dsRNA sequence can also have insertions, deletions and individual point mutations compared with the endogenous β–hydroxylase target sequence and nevertheless causes an efficient reduction of expression. Preferably, the homology is at least 75%, preferably at least 80%, very particularly preferably at least 90%, most preferably 100%, between the "sense" strand of an inhibitory dsRNA and at least a part of the "sense" RNA transcript of an endogenous β–hydroxylase gene, or between the "antisense" strand to the complementary strand of an endogenous β–hydroxylase gene.

A 100% sequence identity between dsRNA and an endogenous β–hydroxylase gene transcript is not absolutely required to cause efficient reduction of the endogenous β–hydroxylase expression. Accordingly, there is the advantage that the method is tolerant toward sequence deviations as can occur as a result of genetic mutations, polymorphisms or evolutionary divergences. For instance it is possible, for example, using the dsRNA which was generated starting from the endogenous β–hydroxylase sequence of the one organism to suppress the endogenous β–hydroxylase expression in another organism. For this purpose, the dsRNA preferably comprises sequence

regions of endogenous β–hydroxylase gene transcripts which correspond to conserved regions. Said conserved regions can readily be derived from sequence comparisons.

Alternatively, an "essentially identical" dsRNA can also be defined as a nucleic acid sequence which is capable of hybridizing with a part of an endogenous β–hydroxylase gene transcript (for example in 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA at 50°C or 70°C for 12 to 16 h).

"Essentially complementary" means that the "antisense" RNA strand can also have insertions, deletions and also individual point mutations compared with the complement of the "sense" RNA strand. Preferably, the homology is at least 80%, preferably at least 90%, very particularly preferably at least 95%, most preferably 100%, between the "antisense" RNA strand and the complement of the "sense" RNA strand.

In a further embodiment, the endogenous β–hydroxylase-dsRNA comprises

a) a "sense" RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of the "sense" RNA transcript of the promoter region of an endogenous β–hydroxylase gene, and

b) an "antisense" RNA strand which is essentially, preferably completely, complementary to the RNA "sense" strand under a).

The corresponding nucleic acid construct which is preferably to be used for transforming of the plants comprises

a) a "sense" DNA strand which is essentially identical to at least a part of the promoter region of an endogenous β–hydroxylase gene, and

b) an "antisense" DNA strand which is essentially, preferably completely, complementary to the DNA "sense" strand under a).

To produce the endogenous β–hydroxylase sequences for reducing the endogenous β–hydroxylase activity, particularly preferably, in particular for *Tagetes erecta,* the following partial sequences are used:

SEQ ID NO: 141: sense fragment of the 5' terminal region of the endogenous β–hydroxylase

SEQ ID NO: 142: antisense fragment of the 5' terminal region of the endogenous β–hydroxylase

The dsRNA can consist of one or more strands of polyribonucleotides. Of course, to achieve the same purpose, a plurality of individual dsRNA molecules each of which comprises one of the above-defined ribonucleotide sequence sections, can also be introduced into the cell or the organism.

The double-stranded dsRNA structure can be formed starting from two complementary separate RNA strands or, preferably, starting from a single, self-complementary RNA strand. In this case, "sense" RNA strand and "antisense" RNA strand are preferably covalently bound to one another in the form of an inverted "repeat".

As described, for example, in WO 99/53050, the dsRNA can also comprise a hairpin structure, by "sense" and "antisense" strand being connected by a linking sequence ("linker"; for example an intron). The self-complementary dsRNA structures are preferred, since they only require the expression of one RNA sequence and always comprise the complementary RNA strands in an equimolar ratio. Preferably, the linking sequence is an intron (for example an intron of the ST-LS1 gene from potato; Vancanneyt GF et al. (1990) Mol Gen Genet 220(2):245-250).

The nucleic acid sequence coding for a dsRNA can contain further elements, for example transcription termination signals or polyadenylation signals.

Further preferred embodiments for reducing the endogenous β-hydroxylase activity result similarly to the above-described preferred embodiments of the reduction of the ε-cyclase activity with exchange of the ε-cyclase by endogenous β-hydroxylase.

Particularly preferably in the inventive method, use is made of genetically modified plants having the following combinations of genetic modifications:

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves and an elevated hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves and an elevated β-cyclase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves and a reduced ε-cyclase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves and an elevated hydroxylase activity and an elevated β-cyclase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves and an elevated hydroxylase activity and a reduced ε-cyclase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves and an elevated β-cyclase activity and a reduced ε-cyclase activity, and also

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves and an elevated hydroxylase activity and an elevated β-cyclase activity and a reduced ε-cyclase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity and an elevated β-cyclase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity and an elevated hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, an elevated β-cyclase activity and an elevated hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, an elevated β-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves and an elevated β-cyclase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity and at least one further elevated activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate D-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, in comparison with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity and an elevated hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity and an elevated β-cyclase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity and an elevated hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated hydroxylase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, an elevated β-cyclase activity, an elevated hydroxylase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity and at least one further elevated activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate D-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity, an elevated hydroxylase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity and an elevated hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated hydroxylase activity and at least one further elevated activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate D-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, a reduced endogenous β-hydroxylase activity and at least one further elevated activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate D-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, an elevated β-cyclase activity, an elevated hydroxylase activity and at least one further elevated activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase

activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, an elevated β-cyclase activity, a reduced endogenous β-hydroxylase activity and at least one further elevated activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate D-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity and an elevated hydroxylase activity and a reduced β-hydroxylase activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity, an elevated hydroxylase activity and at least one further elevated activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate D-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity, a reduced endogenous β-hydroxylase activity and at least one further elevated activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-

phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate D-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity.

Particularly preferred genetically modified plants, compared with the wild type, have an elevated or induced ketolase activity in flower leaves, an elevated β-cyclase activity and an elevated hydroxylase activity,

the elevated ketolase activity being induced by introducing nucleic acids which code for a protein comprising the amino acid sequence SEQ ID NO: 2 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which sequence has an identity of at least 20% at the amino acid level with the sequence SEQ ID NO: 2 and has the enzymatic property of a ketolase,

the elevated β-cyclase activity being induced by introducing nucleic acid coding for a β-cyclase comprising the amino acid sequence SEQ ID NO: 110 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which sequence has an identity of at least 20% at the amino acid level with the sequence SEQ ID NO: 110,

and the elevated hydroxylase activity being induced by introducing nucleic acids coding for a hydroxylase comprising the amino acid sequence SEQ ID NO: 108 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which sequence has an identity of at least 20% at the amino acid level with the sequence SEQ ID NO: 108.

Particularly preferred genetically modified plants have, compared with the wild type, an elevated or induced ketolase activity in flower leaves, a reduced ε-cyclase activity, an elevated β-cyclase activity, an elevated hydroxylase activity and a reduced endogenous β-hydroxylase activity,

the elevated ketolase activity being induced by introducing nucleic acids which code for a protein comprising the amino acid sequence SEQ ID NO: 2 or a sequence derived

from this sequence by substitution, insertion or deletion of amino acids, which sequence has an identity of at least 20% at the amino acid level with the sequence SEQ ID NO: 2 and which has the enzymatic property of a ketolase,

the elevated β-cyclase activity being induced by introducing nucleic acid coding for a β-cyclase comprising the amino acid sequence SEQ ID NO: 110 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which sequence has an identity of at least 20% at the amino acid level with the sequence SEQ ID NO: 100,

the elevated hydroxylase activity being induced by introducing nucleic acids coding for a hydroxylase comprising the amino acid sequence SEQ ID NO: 108 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids, which sequence has an identity of at least 20% at the amino acid level with the sequence SEQ ID NO: 108, and the reduced ε-cyclase activity and a reduced endogenous β-hydroxylase activity being induced in accordance with the above-described preferred embodiments.

These genetically modified plants of the genus Tagetes can, as described hereinafter, be produced, for example by introducing individual nucleic acid constructs (expression cassettes) or by introducing multiple constructs which comprise up to two, three or four of the described activities.

Hereinafter, the production of genetically modified plants having elevated or induced ketolase activity in flower leaves is described by way of example. The elevation of further activities, for example the hydroxylase activity and/or the β-cyclase activity and/or the HMG-CoA reductase activity and/or the (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity and/or the 1-deoxy-D-xylose-5-phosphate synthase activity and/or the 1-deoxy-D-xylose-5-phosphate reductoisomerase activity and/or the isopentenyl-diphosphate D-isomerase activity and/or the geranyl-diphosphate synthase activity and/or the farnesyl-diphosphate synthase activity and/or the geranylgeranyl-diphosphate synthase activity and/or the phytoene synthase activity and/or the phytoene desaturase activity and/or the zeta-carotene desaturase activity and/or the crtISO activity and/or the FtsZ activity and/or the MinD activity, can be achieved in a similar manner using nucleic acid sequences coding for a hydroxylase or β-cyclase or

nucleic acids coding for an HMG-CoA reductase and/or nucleic acids coding for an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or nucleic acids coding for a 1-deoxy-D-xylose-5-phosphate synthase and/or nucleic acids coding for a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or nucleic acids coding for an isopentenyl-diphosphate D-isomerase and/or nucleic acids coding for a geranyl-diphosphate synthase and/or nucleic acids coding for a farnesyl-diphosphate synthase and/or nucleic acids coding for a geranylgeranyl-diphosphate synthase and/or nucleic acids coding for a phytoene synthase and/or nucleic acids coding for a phytoene desaturase and/or nucleic acids coding for a zeta-carotene desaturase and/or nucleic acids coding for a crtIso protein and/or nucleic acids coding for an FtsZ protein and/or nucleic acids coding for a MinD protein instead of nucleic acid sequences coding for a ketolase. The reduction of further activities, for example the reduction of the ε-cyclase activity, or of the endogenous β-hydroxylase activity can be performed in a similar manner using anti-ε-cyclase nucleic acid sequences or ε-cyclase inverted-repeat nucleic acid sequence or using anti-endogenous β-hydroxylase nucleic acid sequences or endogenous β-hydroxylase inverted-repeat nucleic acid sequences instead of nucleic acid sequences coding for a ketolase. The transformation can take place in the combinations of genetic changes, individually or by multiple constructs.

The transgenic plants of the genus Tagetes are produced, preferably, by transforming the starting plants using a nucleic acid construct which comprises the above-described nucleic acids coding for a ketolase which are functionally linked to one or more regulatory signals which ensure transcription and translation in plants.

These nucleic acid constructs in which the coding nucleic acid sequences are functionally linked to one or more regulatory signals which ensure the transcription and translation in plants are also termed hereinafter expression cassettes.

Preferably, the regulatory signals comprise one or more promoters which ensure transcription and translation in plants.

The expression cassettes contain regulatory signals, that is to say regulatory nucleic acid sequences which control the expression of the coding sequence in the host cell. According to a preferred embodiment, an expression cassette comprises upstream, that is to say at the 5' end of the coding sequence, a promoter, and downstream, that is

to say at the 3' end, a polyadenylation signal and if appropriate further regulatory elements which are operationally linked to the coding sequence in-between from at least one of the above-described genes. An operational link is taken to mean the sequential arrangement of promoter, coding sequence, terminator and if appropriate further regulatory elements in such a manner that each of the regulatory elements can fulfill its function in the proper manner in the expression of the coding sequence.

Hereinafter, by way of example, the preferred nucleic acid constructs, expression cassettes and vectors for plants of the genus Tagetes and methods for producing transgenic plants of the genus Tagetes, and also the transgenic plants of the genus Tagetes themselves, are described.

The sequences which are preferred for the operational link, that are not restricted thereto, are targeting sequences for ensuring subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrion, in the endoplasmatic reticulum (ER), in the cell nucleus, in oil bodies or other compartments and translation enhancers such as the 5' lead sequence from tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693-8711).

As promoters of the expression cassette, in principle, any promoter is suitable which can control the expression of foreign genes in plants.

"Constitutive" promoter means those promoters which ensure expression in numerous, preferably all, tissues over a very great period of plant development, preferably at all time points of plant development.

Preferably, use is made of in particular a plant promoter or a promoter which originates from a plant virus. In particular, preference is given to the promoter of the 35S transcript of the CaMV cauliflower mosaic virus (Franck et al. (1980) Cell 21:285-294; Odell et al. (1985) Nature 313:810-812; Shewmaker et al. (1985) Virology 140:281-288; Gardner et al. (1986) Plant Mol Biol 6:221-228) or the 19S CaMV promoter (US 5,352,605; WO 84/02913; Benfey et al. (1989) EMBO J 8:2195-2202).

A further suitable constitutive promoter is the pds promoter (Pecker et al. (1992) Proc. Natl. Acad. Sci USA 89:4962-4966) or the "Rubisco small subunit (SSU)" promoter (US 4,962,028), the LeguminB promoter (GenBank Acc. No. X03677), the promoter of

the nopalin synthase from Agrobacterium, the TR double promoter, the OCS (octopin synthase) promoter from Agrobacterium, the ubiquitin promoter (Holtorf S et al. (1995) Plant Mol Biol 29:637-649), the ubiquitin 1 promoter (Christensen et al. (1992) Plant Mol Biol 18:675-689; Bruce et al. (1989) Proc Natl Acad Sci USA 86:9692-9696), the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (US 5,683,439), the promoters of the vacuolar ATPase subunits or the promoter of a proline-rich protein from wheat (WO 91/13991), the Pnit promoter (Y07648.L, Hillebrand et al. (1998), Plant. Mol. Biol. 36, 89-99, Hillebrand et al. (1996), Gene, 170, 197-200) and also further promoters of genes, the constitutive expression of which in plants is known to those skilled in the art.

The expression cassettes can also comprise a chemically inducible promoter (review article: Gatz et al. (1997) Annu Rev Plant Physiol Plant Mol Biol 48:89-108), by which the expression of the ketolase gene in the plant can be controlled at a defined time point. Such promoters, for example the PRP1 promoter (Ward et al. (1993) Plant Mol Biol 22:361-366), salicylic acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP 0 388 186), a tetracycline-inducible promoter (Gatz et al. (1992) Plant J 2:397-404), an abscisic acid-inducible promoter (EP 0 335 528) and an ethanol- or cyclohexanone-inducible promoter (WO 93/21334) can likewise be used.

Furthermore, promoters are preferred which are induced by biotic or abiotic stress, for example the pathogen-inducible promoter of the PRP1 gene (Ward et al. (1993) Plant Mol Biol 22:361-366), the heat-inducible hsp70 or hsp80 promoter from tomato (US 5,187,267), the cold-inducible alpha-amylase promoter from potato (WO 96/12814), the light-inducible PPDK promoter or the wound-induced pinII promoter (EP 375091).

Pathogen-inducible promoters comprise those of genes which are induced as a result of pathogen attack, for example genes of PR proteins, SAR proteins, β-1,3-glucanase, chitinase etc. (for example Redolfi et al. (1983) Neth J Plant Pathol 89:245-254; Uknes, et al. (1992) The Plant Cell 4:645-656; Van Loon (1985) Plant Mol Viral 4:111-116; Marineau et al. (1987) Plant Mol Biol 9:335-342; Matton et al. (1987) Molecular Plant-Microbe Interactions 2:325-342; Somssich et al. (1986) Proc Natl Acad Sci USA 83:2427-2430; Somssich et al. (1988) Mol Gen Genetics 2:93-98; Chen et al. (1996)

Plant J 10:955-966; Zhang and Sing (1994) Proc Natl Acad Sci USA 91:2507-2511; Warner, et al. (1993) Plant J 3:191-201; Siebertz et al. (1989) Plant Cell 1:961-968(1989).

Those which are also comprised are wound-inducible promoters such as that of the pinII gene (Ryan (1990) Ann Rev Phytopath 28:425-449; Duan et al. (1996) Nat Biotech 14:494-498), of the wun1 and wun2 gene (US 5,428,148), of the win1 and win2 gene (Stanford et al. (1989) Mol Gen Genet 215:200-208), of the systemin (McGurl et al. (1992) Science 225:1570-1573), of the WIP1 gene (Rohmeier et al. (1993) Plant Mol Biol 22:783-792; Ekelkamp et al. (1993) FEBS Letters 323:73-76), of the MPI gene (Corderok et al. (1994) The Plant J 6(2):141-150) and the like.

Further suitable promoters are, for example, fruit-ripening-specific promoters, for example the fruit-ripening-specific promoter from tomato (WO 94/21794, EP 409 625). Development-dependent promoters include in part the tissue-specific promoters, since individual tissues, of course, form in a development-dependent manner.

Furthermore, in particular those promoters are preferred which ensure the expression in tissues or plant parts in which, for example, the biosynthesis of ketocarotenoids, or precursors thereof takes place. Preference is given to, for example, promoters having specificities for anthers, ovaries, petals, sepals, flowers, leaves, stems and roots and combinations thereof.

Tuber-, storage root- or root-specific promoters are, for example, the patatin promoter class I (B33) or the promoter of the cathepsin D inhibitor from potatoes.

Leaf-specific promoters are, for example, the promoter of the cytosol FBPase from potatoes (WO 97/05900), the SSU promoter (small subunit) of rubisco (ribulose-1,5-bisphosphate carboxylase) or the ST-LSI promoter from potatoes (Stockhaus et al. (1989) EMBO J 8:2445-2451).

Flower-specific promoters are, for example, the phytoene synthase promoter (WO 92/16635) or the promoter of the P-rr gene (WO 98/22593) or the AP3 promoter from Arabidopsis thaliana (see Example 1).

Anther-specific promoters are; for example, the 5126 promoter (US 5,689,049, US 5,689,051), the glob-1 promoter or the g-zein promoter.

Further promoters suitable for expression in plants are described in Rogers et al. (1987) Meth in Enzymol 153:253-277; Schardl et al. (1987) Gene 61:1-11 and Berger et al. (1989) Proc Natl Acad Sci USA 86:8402-8406).

All of the promoters described in the present application generally make possible the expression of the ketolase in flower leaves of the inventive plants.

Particular preference in the inventive method is given to constitutive flower-specific, and in particular flower leaf-specific, promoters.

An expression cassette is preferably produced by fusion of a suitable promoter with an above-described nucleic acid coding for a ketolase and preferably a nucleic acid which is inserted between promoter and nucleic acid sequence and codes for a plastid-specific transit peptide and also with a polyadenylation signal according to customary recombination and cloning techniques, as described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and also in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

The nucleic acids which are preferably inserted and code for a plastid transit peptide ensure localization in plastids, and in particular in chromoplasts.

Expression cassettes can also be used, the nucleic acid sequence of which codes for a ketolase fusion protein, a part of the fusion protein being a transit peptide which controls the translocation of the polypeptide. Preference is given, for the chromoplasts, to specific transit peptides which, after translocation of the ketolase into the chromoplasts, are enzymatically cleaved from the ketolase part.

In particular, preference is given to the transit peptide which is derived from the plastid *Nicotiana tabacum* transketolase or from another transit peptide (for example the

transit peptide of the small subunit of rubisco (rbcS) or the ferredoxin NADP oxidoreductase, but also the isopentenyl-pyrophosphate isomerase-2 or its functional equivalent.

Particular preference is given to nucleic acid sequences of three cassettes of the plastid transit peptide of the plastid transketolase from tobacco in three reading frames as KpnI/BamHI fragments having an ATG codon in the NcoI cut site:

pTP09

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTC
GTTCTGTCCCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTC
ACTTTTTCCGGCCTTAAATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCC
TTCCTCCGCCGCCGCCGCCGCCGTCGTAAGGTCACCGGCGATTCGTGCCTCAGC
TGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGATCC_BamHI

pTP10

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTC
GTTCTGTCCCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTC
ACTTTTTCCGGCCTTAAATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCC
TTCCTCCGCCGCCGCCGCCGCCGTCGTAAGGTCACCGGCGATTCGTGCCTCAGC
TGCAACCGAAACCATAGAGAAAACTGAGACTGCGCTGGATCC_BamHI

pTP11

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTC
GTTCTGTCCCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTC
ACTTTTTCCGGCCTTAAATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCC
TTCCTCCGCCGCCGCCGCCGCCGTCGTAAGGTCACCGGCGATTCGTGCCTCAGC
TGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGGATCC_BamHI

Further examples of a plastid transit peptide are the transit peptide of the plastid isopentenyl-pyrophosphate isomerase-2 (IPP-2) from Arabidopsis thaliana and the transit peptide of the small subunit of ribulose-bisphosphate carboxylase (rbcS) from peas (Guerineau, F, Woolston, S, Brooks, L, Mullineaux, P (1988) An expression

cassette for targeting foreign proteins into the chloroplasts. Nucl. Acids Res. 16: 11380).

The inventive nucleic acids can be prepared synthetically or produced naturally or comprise a mixture of synthetic and natural nucleic acid constituents, and can also consist of various heterologous gene sections of various organisms.

Preference is given to, as described above, synthetic nucleotide sequences having codons which are preferred by plants of the genus Tagetes. These codons preferred by plants can be determined from codons having the highest protein frequency which are expressed in the most plant species of interest.

In the preparation of an expression cassette, various DNA fragments can be manipulated to obtain a nucleotide sequence which expediently reads in the correct direction and which is equipped with a correct reading frame. For connecting the DNA fragments to one another, adapters or linkers can be attached to the fragments.

Expediently, the promoter and terminator regions can be provided in the transcription direction with a linker or polylinker which comprises one or more restriction sites for the insertion of this sequence. Generally, the linker has from 1 to 10, usually from 1 to 8, preferably from 2 to 6, restriction sites. Generally, the linker, within the regulatory regions, has a size of less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter can either be native or homologous but also foreign or heterologous to the host plant. The expression cassette preferably comprises in the 5'-3' transcription direction the promoter, a coding nucleic acid sequence or a nucleic acid construct and a region for the transcriptional termination. Various termination regions are exchangeable for one another as desired.

Examples of a terminator are the 35S terminator (Guerineau et al. (1988) Nucl Acids Res. 16: 11380), the nos terminator (Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM. Nopaline synthase: transcript mapping and DNA sequence. J Mol Appl Genet. 1982;1(6):561-73) or the ocs terminator (Gielen, J, de Beuckeleer, M, Seurinck, J, Debroek, H, de Greve, H, Lemmers, M, van Montagu, M, Schell, J (1984) The complete sequence of the TL-DNA of the Agrobacterium tumefaciens plasmid pTiAch5. EMBO J. 3: 835-846).

Furthermore, use can be made of manipulations which provide matching restriction cut sites or which remove the excess DNA or restriction cut sites. Where insertions, deletions or substitutions, for example transitions and transversions, come into question, *in vitro* mutagenesis, "primer repair", restriction or ligation can be used.

With suitable manipulations, for example restriction, "chewing-back" or filling-in of overhangs for "blunt ends", complementary ends of the fragments for ligation can be made available.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which essentially correspond to T-DNA polyadenylation signals from Agrobacterium tumefaciens, in particular gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984), 835 ff) or functional equivalents.

The transfer of foreign genes to the genome of a plant is termed transformation.

For this, methods known per se can be utilized for the transformation and regeneration of plants from plant tissues or plant cells for transient or stable transformation.

Suitable methods for the transformation of plants are protoplast transformation by polyethylene glycol-induced DNA uptake, the biolistic method using the gene gun, what is termed the particle bombardment method, electroporation, the incubation of dry embryos in DNA-containing solution, microinjection and the above-described gene transfer mediated by Agrobacterium. Said methods are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, published by S.D. Kung and R. Wu, Academic Press (1993), 128-143 and also in Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225).

Preferably, the construct to be expressed is cloned into a vector which is suitable for transforming Agrobacterium tumefaciens, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711) or particularly preferably pSUN2, pSUN3, pSUN4 or pSUN5 (WO 02/00900).

Agrobacteria transformed by an expression plasmid can be used in a known manner for the transformation of plants, for example by bathing wounded leaves or leaf pieces in an Agrobacteria solution and then culturing them in suitable media.

For the preferred production of genetically modified plants, hereinafter also termed transgenic plants, the fused expression cassette which expresses a ketolase is cloned into a vector, for example pBin19, or in particular pSUN2, which is suitable for being transformed in *Agrobacterium tumefaciens*. Agrobacteria transformed using such a vector can then be used in a known manner for transforming plants, in particular cultivated plants by, for example, bathing wounded leaves or leaf pieces in an Agrobacteria solution and then culturing them in suitable media.

The transformation of plants by Agrobacteria is disclosed, inter alia, by F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, published by S.D. Kung and R. Wu, Academic Press, 1993, pp. 15-38. From the transformed cells of the wounded leaves or leaf pieces, in a known manner, transgenic plants can be regenerated which comprise a gene which is integrated into the expression cassette and is for the expression of a nucleic acid coding for a ketolase.

For the transformation of a host plant of the genus Tagetes having a nucleic acid coding for a ketolase, an expression cassette is incorporated as insertion into a recombinant vector, the vector DNA of which comprises additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described, inter alia, in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), Chapters 6/7, pp. 71-119 (1993).

Using the above-cited recombination and cloning techniques, the expression cassettes can be cloned into suitable vectors which allow their multiplication, for example in *E. coli*. Suitable cloning vectors are, inter alia, pJIT117 (Guerineau et al. (1988) Nucl. Acids Res.16 :11380), pBR332, pUC series, M13mp series and pACYC184. Particularly suitable vectors are binary vectors which can replicate not only in *E. coli* but also in Agrobacteria.

Depending on the choice of promoter, the expression can be performed in the flower leaves constitutively or preferably specifically.

The inventive genetically modified plants of the genus Tagetes, compared with the wild type, have a content of astaxanthin, in particular in petals.

As mentioned above, the invention relates to the use of astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes for oral administration to animals.

In a preferred embodiment, the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes are used for pigmenting animals and the corresponding animal products.

Astaxanthin-containing extracts of astaxanthin-containing plants or plant parts are preferably taken to mean solutions comprising astaxanthin which have been produced by extraction from astaxanthin-containing plants or plant parts with at least one suitable solvent. Depending on solvent used and further chemical and physical purification methods used, the astaxanthin can be present in the extract in any desired degrees of purity. It is advantageous to prepare the astaxanthin-containing plants or plant parts appropriately before extraction, for example to dry the plants or plant parts and comminute them, the sequence being optional.

Astaxanthin can be extracted from the astaxanthin-containing plants or plant parts, which if appropriate have been previously dried and/or comminuted, by organic solvents, for example acetone, hexane, methylene chloride, methyl tertiary-butyl ether, or by solvent mixtures such as ethanol/hexane or acetone/hexane. By means of differing mixing ratios of the solvents, owing to the differing polarity, the extraction effect can be varied. By means of such an extraction, astaxanthin may be enriched at high concentration.

The purity of astaxanthin can be further increased by then extracting astaxanthin by shaking and chromatographic separation of the mixture. Astaxanthin is generally present as a mixture of mono- and diesters, usually as esters of palmitic acid.

"Pigmenting" according to the invention is preferably taken to mean the intensifying or initiating of a color, at least of a part of an animal or animal product of the pigmented animal, compared with the non-pigmented animal. Astaxanthin-containing pigments generally pigment and initiate or intensify a pink to pink-red color note.

Preferred animals which can be pigmented by the inventive oral administration are animals selected from the group fish, crustaceae or birds, in particular Galliformes and Anatridae.

Preferred fish are Salmonids, in particular salmon or trout.

Preferred Crustaceae are shrimps or crabs.

Preferred Galliformes are chickens, ducks or geese.

Preferred Anatridae is flamingo.

Depending on the pigmented animal, preferably, pigmented animal products are taken to mean, in particular meat for salmon or trout, skin for chickens, ducks or geese, feathers for chickens, ducks, geese or flamingo, and egg or egg yolk for chickens, ducks or geese.

The oral administration of the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes to animals can be performed directly or via oral administration of animal feed preparations to which the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes have been admixed in advance.

In a preferred embodiment, the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes are admixed to animal feed preparations and the animal feed preparation is orally administered to animals.

It is advantageous to process the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, before the admixture to animal feed preparations, into a form which makes possible admixture to corresponding animal feed preparations and preferably leads to high stability and bioavailability of astaxanthin in the respective field of use.

Depending on the animal to which the oral administration is to take place and thus depending on animal feed preparation, various processing steps can be advantageous for this.

For astaxanthin-containing plants or parts of plants of the genus Tagetes it is advantageous in this embodiment to dry and/or comminute the astaxanthin-containing plants or parts of plants, in particular flower heads and petals. Particularly preferably, the astaxanthin-containing plants or parts of plants of the genus Tagetes are present in pulverulent form.

Every embodiment however arranged of the astaxanthin-containing plants or parts of plants of the genus Tagetes, whether processed or unprocessed, can be admixed in a manner known per se to animal feed preparations.

For astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, in this embodiment, various processing steps are advantageous.

The astaxanthin-containing extracts can, provided that the solvents still present are physiologically harmless for the corresponding animals, be admixed directly to the animal feed preparation.

The extracts, after evaporating off the solvents still present, can be used in the form of astaxanthin-containing powders or oils.

The resultant astaxanthin-containing powders or oils can, for example, be incorporated into fish oil, applied to pulverulent carrier materials, for example wheat flour or grated Tagetes petals, or included in alginates, gelatin or lipids.

The astaxanthin-containing extracts or processed extracts are thus preferably in liquid or pulverulent form.

Every embodiment however arranged of the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, whether processed or unprocessed, can be admixed in a manner known per se to animal feed preparations.

The invention therefore also relates to animal feed preparations comprising astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes.

The invention further relates to a method for producing animal feed preparations by combining astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes and customary animal feedstuffs.

A preferred embodiment of the method comprises processing the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, before the combination with animal feedstuffs, into a form which makes possible combination with animal feedstuffs.

For example, for fish, the fish feed preparations can comprise further customary fish feed components, for example fish meal and/or other proteins, oils, for example fish oils, cereals, vitamins, minerals, preservatives and if appropriate medicaments in customary amounts.

A typical fish feed formula for trout is composed, for example, from the following components:

Components	*% by weight*	Weight for 500 kg *kg*
Fish meal	30.00	150.00
Full fat soybeans	20.00	100.00
Pregelatinized wheat starch	18.00	90.00
Vitamin premix	0.80	4.00
Choline chloride (50%)	0.20	1.00
Wheat gluten	20.00	100.00
Sipernat 50S	3.00	15.00
Fish oil	8.00	40.00

A typical fish feed formula for salmon is composed, for example, of the following components:

Components	*% by weight*
Fish meal	75.00
Plant protein	5.00
Cereal	7.80
Vitamins/minerals	1.00
Antioxidants/preservatives	0.20
Fish oil	11.00

In one embodiment, the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts are admixed to the animal feed preparations preferably in dried and comminuted pulverulent form.

The resultant animal feed preparations comprising astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes can, in the case of fish feed,

be, for example in a manner known per se, pelleted, or particularly advantageously extruded.

In a preferred embodiment, the astaxanthin-containing extracts are admixed to the animal feed preparations, preferably in liquid form. This is advantageous, in particular, in the production of extruded fish feed preparations. The extrusion process leads to extrusion stress on the sensitive substances, for example astaxanthin, which can lead to an astaxanthin loss. Extrusion stress is primarily the action of mechanical forces (kneading, shearing, pressure, etc.), but also hydrothermal stress, caused by additions of water and steam, and also oxidative stress may be observed.

To avoid the astaxanthin losses occurring as a result of the above-described extrusion process, liquid astaxanthin-containing extracts may be applied under vacuum after the extrusion and drying process by the PPA technique (*post-pelleting application*).

In a further, preferred embodiment, the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes are orally administered directly to animals.

It is advantageous to process the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, before the administration, into a form which makes possible direct oral administration to animals and preferably leads to a high stability and bioavailability of astaxanthin in the respective field of use.

Depending on the animal to which the oral administration is to take place, and thus depending on animal feed preparation, various processing steps can be advantageous for this.

For astaxanthin-containing plants or parts of plants of the genus Tagetes, it is advantageous in this embodiment to dry and/or comminute the astaxanthin-containing plants or parts of plants, in particular flower heads and petals. Particularly preferably, the astaxanthin-containing plants or parts of plants of the genus Tagetes are present in pulverulent form.

Every embodiment, however arranged, of the astaxanthin-containing plants or parts of plants of the genus Tagetes, whether processed or unprocessed, can be orally administered to animals in a manner known per se.

For astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, various processing steps are advantageous in this embodiment.

The astaxanthin-containing extracts, provided that the solvents still present are physiologically harmless for the respective animals, can be administered orally directly to animals.

The extracts can be administered, after evaporation of the solvents which are still present, in the form of astaxanthin-containing powders or oils.

The resultant astaxanthin-containing powders or oils can be incorporated, for example, into fish oil, can be applied to pulverulent support materials, for example wheat flour or grated Tagetes petals, or included in alginates, gelatin or lipids.

The astaxanthin-containing extracts or processed extracts are thus preferably in liquid or pulverulent form.

Every embodiment however arranged of the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, whether processed or unprocessed, can be administered orally to animals in a manner known per se.

The invention therefore also relates to pigmenting agents comprising astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, in which case the astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes can if appropriate be processed as described above.

In a preferred embodiment, the pigmenting agents consist of astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, in which case the

astaxanthin-containing plants or parts of plants of the genus Tagetes or the astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes can if appropriate be processed as described above.

In particularly preferred pigmenting agents, the plant parts used are flower heads or petals.

The invention further relates to a method for pigmenting animals or animal products by oral administration of astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes to animals.

The invention further relates to a method for producing pigmented animals or animal products by oral administration of astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes to animals.

The invention further relates to the use of astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes as animal feed or animal feed additive.

The pigmenting agents comprising astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, or animal feedstuffs comprising these pigmenting agents further have the advantage of a high storage stability and bioavailability of the pigment astaxanthin.

The invention will now be described by the following examples, but is not restricted thereto:

Example I

Production of astaxanthin-containing genetically modified plants of the genus Tagetes

General experimental conditions:

Sequence analysis of recombinant DNA

Recombinant DNA molecules are sequenced using a laser fluorescence DNA-sequencer from Licor (distributed by MWG Biotech, Ebersbach) by the method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463-5467).

Example I.1:
Amplification of a cDNA which codes for the entire primary sequence of the ketolase from *Haematococcus pluvialis* Flotow em. Wille

The cDNA which codes for the ketolase from *Haematococcus pluvialis* was amplified by means of PCR from *Haematococcus pluvialis* (strain 192.80 of the Collection of Algal Cultures of the University of Göttingen) suspension culture.

For the preparation of total RNA from a suspension culture of *Haematococcus pluvialis* (strain 192.80) which had been grown for 2 weeks in indirect daylight at room temperature in *Haematococcus* medium (1.2 g/l of sodium acetate, 2 g/l of yeast extract, 0.2 g/l of $MgCl_2 \cdot 6H_2O$, 0.02 $CaCl_2 \cdot 2H_2O$; pH 6.8; after autoclaving, addition of 400 mg/l of L-asparagine, 10 mg/l of $FeSO_4 \cdot H_2O$), the cells were harvested, frozen in liquid nitrogen and ground in the mortar. 100 mg of the frozen pulverized algae cells were then transferred to a reaction vessel and taken up in 0.8 ml of Trizol buffer (LifeTechnologies). The suspension was extracted with 0.2 ml of chloroform. After centrifugation for 15 minutes at 12 000 g, the aqueous supernatant was taken off and transferred to a new reaction vessel and extracted with one volume of ethanol. The RNA was precipitated with one volume of isopropanol, washed with 75% ethanol and the pellet was dissolved in DEPC water (overnight incubation of water with 1/1000 volume of diethyl pyrocarbonate at room temperature, then autoclaved). The RNA concentration was determined photometrically.

For the cDNA synthesis, 2.5 μg of total RNA were denatured for 10 min at 60°C, cooled on ice for 2 min and transcribed to cDNA by means of a cDNA kit (Ready-to-go-you-prime-beads, Pharmacia Biotech) according to the manufacturer's instructions, using an antisense-specific primer (PR1 SEQ ID NO: 29).

The nucleic acid coding for a ketolase from *Haematococcus pluvialis* (strain 192.80) was amplified by means of the polymerase chain reaction (PCR) from *Haematococcus*

pluvialis using a sense-specific primer (PR2 SEQ ID NO: 30) and an antisense-specific primer (PR1 SEQ ID NO: 29).

The PCR conditions were as follows:

The PCR for amplification of the cDNA which codes for a ketolase protein consisting of the entire primary sequence was carried out in a 50 ml reaction mix in which the following were present:

- 4 ml of a *Haematococcus pluvialis* cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM PR1 (SEQ ID NO: 29)
- 0.2 mM PR2 (SEQ ID NO: 30)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 25.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	53°C	2 minutes
	72°C	3 minutes
1×	72°C	10 minutes

The PCR amplification using SEQ ID NO: 29 and SEQ ID NO: 30 resulted in a 1155 bp fragment which codes for a protein consisting of the entire primary sequence (SEQ ID NO: 22). Using standard methods, the amplicon was cloned into the PCR cloning vector pGEM-Teasy (Promega) and the clone pGKETO2 was obtained.

Sequencing the clone pGKETO2 having the T7 and the SP6 primer confirmed a sequence which only differs from the published sequence X86782 in the three codons 73, 114 and 119, each in one base. These nucleotide replacements were reproduced in an independent amplification experiment and thus represent the nucleotide

sequence in the *Haematococcus pluvialis* strain 192.80 used (Figures 1 and 2, sequence comparisons).

This clone was therefore used for the cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380). The cloning was performed by isolating the 1027 bp SpHI fragment from pGEM-Teasy and ligation into the SpHI-cut vector pJIT117. The clone which comprises the *Haematococcus pluvialis* ketolase in the correct orientation as N-terminal translational fusion with the rbcs transit peptide is called pJKETO2.

Example I.2:
Amplification of a cDNA which codes for the ketolase from *Haematococcus pluvialis* Flotow em. Wille having an N terminus shortened by 14 amino acids

The cDNA which codes for the ketolase from *Haematococcus pluvialis* (strain 192.80) having an N terminus shortened by 14 amino acids was amplified by means of PCR from *Haematococcus pluvialis* suspension culture (strain 192.80 of the Collection of Algal Cultures of the University of Göttingen).

Total RNA was prepared from a suspension culture of *Haematococcus pluvialis* (strain 192.80) as described in Example 1.

The cDNA synthesis was performed as described under Example 1.

The nucleic acid coding for a ketolase from *Haematococcus pluvialis* (strain 192.80) having an N terminus shortened by 14 amino acids was amplified by means of the polymerase chain reaction (PCR) from *Haematococcus pluvialis* using a sense-specific primer (PR3 SEQ ID NO: 31) and an antisense-specific primer (PR1 SEQ ID NO: 29).

The PCR conditions were as follows:

The PCR for amplification of the cDNA which codes for a ketolase protein having an N terminus shortened by 14 amino acids was performed in a 50 ml reaction mix in which the following were present:

- 4 ml of a *Haematococcus pluvialis* cDNA (prepared as described above)

- 0.25 mM dNTPs
- 0.2 mM PR1 (SEQ ID NO: 29)
- 0.2 mM PR3 (SEQ ID NO: 31)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 25.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	53°C	2 minutes
	72°C	3 minutes
1×	72°C	10 minutes

The PCR amplification using SEQ ID NO: 29 and SEQ ID NO: 31 resulted in a 1111 bp fragment which codes for a ketolase protein in which N-terminal amino acids (positions 2-16) are replaced by a single amino acid (leucine).

The amplicon was cloned using standard methods into the PCR cloning vector pGEM-Teasy (Promega). Sequencing with the primers T7 and SP6 confirmed a sequence identical to sequence SEQ ID NO: 22, in which the 5' region (positions 1-53) of SEQ ID NO: 22 was replaced in the amplicon SEQ ID NO: 24 by a nonamer sequence deviating in the sequence. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

The cloning was performed by isolating the 985 bp SpHI fragment from pGEM-Teasy and ligation with the SpHI-cut vector pJIT117. The clone which comprises the *Haematococcus pluvialis* ketolase having an N terminus shortened by 14 amino acids in the correct orientation as N-terminal translational fusion with the rbcs transit peptide is called pJKETO3.

Example I.3:

Amplification of a cDNA which codes for the ketolase from *Haematococcus pluvialis* Flotow em. Wille (strain 192.80 of the Collection of Algal Cultures of the University of Göttingen) consisting of the entire primary sequence and fused C-terminal myc-Tag

The cDNA which codes for the ketolase from *Haematococcus pluvialis* (strain 192.80) consisting of the entire primary sequence and fused C-terminal myc-Tag was prepared by means of PCR using the plasmid pGKETO2 (described in Example 1) and the primer PR15 (SEQ ID NO: 32). The primer PR15 is composed of an antisense specific 3' region (nucleotides 40 to 59) and an myc-Tag coding 5' region (nucleotides 1 to 39).

The denaturation (5 min at 95°C) and annealing (slow cooling at room temperature to 40°C) of pGKETO2 and PR15 was performed in a 11.5 ml reaction mix in which the following were present:

- 1 mg of pGKETO2 plasmid DNA
- 0.1 mg of PR15 (SEQ ID NO: 32)

The 3' ends were filled in (30 min at 30°C) in a 20 ml reaction mix in which the following were present:

- 11.5 ml of pGKETO2/PR15 annealing reaction (produced as described above)
- 50 mM dNTPs
- 2 ml of 1× Klenow buffer
- 2 U of Klenow enzyme

The nucleic acid coding for a ketolase from *Haematococcus pluvialis* (strain 192.80) consisting of the entire primary sequence and fused C terminal myc-Tag was amplified by means of the polymerase chain reaction (PCR) from *Haematococcus pluvialis* using a sense-specific primer (PR2 SEQ ID NO: 30) and an antisense-specific primer (PR15 SEQ ID NO: 32).

The PCR conditions were as follows:

The PCR for amplification of the cDNA which codes for a ketolase protein having a fused C-terminal myc-Tag was performed in a 50 ml reaction mix in which the following were present:

- 1 ml of an annealing reaction (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR15 (SEQ ID NO: 32)
- 0.2 mM PR2 (SEQ ID NO: 30)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 28.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The PCR amplification with SEQ ID NO: 32 and SEQ ID NO: 30 resulted in a 1032 bp fragment which codes for a protein consisting of the entire primary sequence of the ketolase from *Haematococcus pluvialis* as two-fold translational fusion with the rbcS transit peptide at the N terminus and the myc-Tag at the C terminus.

The amplicon was cloned using standard methods into the PCR cloning vector pGEM-Teasy (Promega). Sequencing with the primers T7 and SP6 confirmed a sequence identical to the sequence SEQ ID NO: 22, where the 3' region (positions 993 to 1155) of SEQ ID NO: 22 was replaced in the amplicon SEQ ID NO: 26 by a deviating sequence of 39 bp. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

The cloning was performed by isolating the 1038 bp EcoRI-SpHI fragment from pGEM-Teasy and ligation with the EcoRI-SpHI-cut vector pJIT117. The ligation produces a translational fusion between the C terminus of the rbcS transit peptide sequence and the N terminus of the ketolase sequence. The clone which comprises the *Haematococcus pluvialis* ketolase having the fused C terminus myc-Tag in the correct

orientation as translational N-terminal fusion with the rbcs transit peptide is called pJKETO4.

Example I.4:

Amplification of a DNA which codes for the entire primary sequence of the ketolase from *Nostoc sp. PCC 7120*

The DNA which codes for the ketolase from *Nostoc PCC 7120* was amplified by means of PCR from *Nostoc PCC 7120* (strain of the "Pasteur Culture Collection of Cyanobacterium").

For the preparation of genomic DNA from a suspension culture of *Nostoc PCC 7120* which had grown for 1 week under constant light with constant shaking (150 rpm) at 25°C in *BG 11* medium (1.5 g/l of $NaNO_3$, 0.04 g/l of $K_2PO_4 \cdot 3H_2O$, 0.075 g/l of $MgSO_4 \cdot H_2O$, 0.036 g/l of $CaCl_2 \cdot 2H_2O$, 0.006 g/l of citric acid, 0.006 g/l of ferric ammonium citrate, 0.001 g/l of EDTA disodium magnesium, 0.04 g/l of Na_2CO_3, 1 ml of trace metal mix A5+Co (2.86 g/l of H_3BO_3, 1.81 g/l of $MnCl_2 \cdot 4H_2O$, 0.222 g/l of $ZnSO_4 \cdot 7H_2O$, 0.39 g/l of $NaMoO_4 \cdot 2H_2O$, 0.079 g/l of $CuSO_4 \cdot 5H_2O$, 0.0494 g/l of $Co(NO_3)_2 \cdot 6H_2O$), the cells were harvested by centrifugation, frozen in liquid nitrogen and pulverized in the mortar.

Protocol for DNA isolation from Nostoc PCC7120:

The bacterial cells were pelleted from a 10 ml liquid culture by centrifugation for 10 minutes at 8000 rpm. The bacterial cells were then crushed and ground in liquid nitrogen using a mortar. The cell material was resuspended in 1 ml of 10 mM Tris HCl (pH 7.5) and transferred to an Eppendorf reaction vessel (2 ml volume). After addition of
100 µl of proteinase K (concentration: 20 mg/ml), the cell suspension was incubated at 37°C for 3 hours. The suspension was then extracted with 500 µl of phenol. After centrifugation for 5 minutes at 13 000 rpm, the upper, aqueous phase was transferred to a new 2 ml Eppendorf reaction vessel. The extraction with phenol was repeated 3 times. The DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of isopropanol, and then washed with 70% ethanol. The DNA

pellet was dried at room temperature, taken up in 25 µl of water and dissolved with heating at 65°C.

The nucleic acid coding for a ketolase from *Nostoc PCC 7120* was amplified by means of the polymerase chain reaction (PCR) from *Nostoc PCC 7120* using a sense-specific primer (NOSTF, SEQ ID No. 87) and an antisense-specific primer (NOSTG, SEQ ID NO. 88).

The PCR conditions were as follows:

The PCR for amplification of the DNA which codes for a ketolase protein consisting of the entire primary sequence was performed in a 50 µl reaction mix in which the following were present:

- 1 µl of a *Nostoc PCC 7120* DNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM NOSTF (SEQ ID No. 87)
- 0.2 mM NOSTG (SEQ ID No. 88)
- 5 µl of 10× PCR buffer (TAKARA)
- 0.25 µl of R Taq polymerase (TAKARA)
- 25.8 µl of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	55°C	1 minute
	72°C	3 minutes
1×	72°C	10 minutes

The PCR amplification with SEQ ID No. 87 and SEQ ID No. 88 resulted in an 805 bp fragment which codes for a protein consisting of the entire primary sequence (SEQ ID No. 89). Using standard methods, the amplicon was cloned into the PCR cloning vector pGEM-T (Promega) and the clone pNOSTF-G was obtained.

Sequencing the clone pNOSTF-G with the M13F and the M13R primer confirmed a sequence which is identical to the DNA sequence of the database entry AP003592. This nucleotide sequence was reproduced in an independent amplification experiment and thus represents the nucleotide sequence in the *Nostoc PCC 7120* used.

This clone pNOSTF-G was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380). The cloning was performed by isolating the 1027 bp SphI fragment from pGEM-T and ligation into the SphI-cut vector pJIT117. The clone which comprises the ketolase of *Nostoc* in the correct orientation as N-terminal translational fusion with the rbcS transit peptide is called pJNOST.

Example I.5:
Production of expression vectors for the constitutive expression of *Haematococcus pluvialis* ketolase in *Tagetes erecta*.

The ketolase from *Haematococcus pluvialis* was expressed in *Tagetes erecta* under the control of the constitutive promoter d35S from CaMV (Franck et al. 1980, Cell 21: 285-294). The expression was performed using the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715).

An expression cassette for the Agrobacterium-mediated transformation of the ketolase from *Haematococcus pluvialis* in Tagetes erecta was produced using the binary vector pSUN5 (WO 02/00900).

For production of the Tagetes expression vector pS5KETO2, the 2.8 Kb SacI-XhoI fragment from pJKETO2 was ligated with the SacI-XhoI-cut vector pSUN5 (Figure 3, construct map). In Figure 3, fragment *d35S* comprises the duplicated 35S promoter (747 bp), fragment *rbcS*, the rbcS transit peptide from pea (204 bp), fragment *KETO2* (1027 bp) the entire primary sequence coding for the *Haematococcus pluvialis* ketolase, fragment *term* (761 bp) the polyadenylation signal of CaMV.

Example I.5A:
Production of expression vectors for the flower-specific expression of the *Haematococcus pluvialis* ketolase in *Tagetes erecta*

The ketolase from *Haematococcus pluvialis* was expressed in *Tagetes erecta* using the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715). The expression was performed under the control of a modified version AP3P of the flower-specific promoter AP3 from *Arabidopsis thaliana* (AL132971: nucleotide region 9298 to 10 200; Hill et al. (1998) Development 125: 1711-1721).

The DNA fragment which comprises the AP3 promoter region -902 to +15 from *Arabidopsis thaliana* was produced by means of PCR using genomic DNA (isolated by standard methods from *Arabidopsis thaliana*) and also the primer PR7 (SEQ ID NO: 33) and PR10 (SEQ ID NO: 36).

The PCR conditions were as follows:

The PCR for amplification of the DNA which comprises the AP3 promoter fragment (-902 to +15) was carried out in a 50 ml reaction mix in which the following were present:

- 100 ng of genomic DNA from *A.thaliana*
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 33)
- 0.2 mM PR10 (SEQ ID NO: 36)
- 5 ml of 10× PCR buffer (Stratagene)
- 0.25 ml of Pfu polymerase (Stratagene)
- 28.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The 922 bp amplicon was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods, and the plasmid pTAP3 was obtained.

Sequencing the clone pTAP3 confirmed a sequence which differs from the published AP3 sequence (AL132971, nucleotide region 9298 to 10 200) only in an insertion (a G in position 9765 of the sequence AL132971) and a base exchange (a G instead of an A in position 9726 of the sequence AL132971). These nucleotide differences were reproduced in an independent amplification experiment and thus represent the actual nucleotide sequence in the *Arabidopsis thaliana* plants used.

The modified version AP3P was produced by means of recombinant PCR using the plasmid pTAP3. The region 10 200 to 9771 was amplified with the primers PR7 (SEQ ID NO: 33) and PR9 (SEQ ID NO: 35) (amplicon A7/9), the region 9526 to 9285 was amplified with PR8 (SEQ ID NO: 34) and PR10 (SEQ ID NO: 36) (amplicon A8/10).

The PCR conditions were as follows:

The PCR reactions for amplification of the DNA fragments which comprise the regions region 10 200-9771 and region 9526 to 9285 of the AP3 promoter were carried out in 50 ml reaction mixes, in which the following were present:

- 100 ng of AP3 amplicon (described above)
- 0.25 mM dNTPs
- 0.2 mM sense primer (PR7 SEQ ID NO: 33 or PR8 SEQ ID NO: 34)
- 0.2 mM antisense primer (PR9 SEQ ID NO: 35 or PR10 SEQ ID NO: 36)
- 5 ml of 10× PCR buffer (Stratagene)
- 0.25 ml of Pfu Taq polymerase (Stratagene)
- 28.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The recombinant PCR comprises annealing of the amplicons A7/9 and A8/10 overlapping over a sequence of 25 nucleotides, completion to give a double strand and subsequent amplification. This produces a modified version of the AP3 promoter, AP3P, in which positions 9670 to 9526 are deleted. The denaturation (5 min at 95°C) and annealing (slow cooling at room temperature to 40°C) of both amplicons A7/9 and A8/10 was carried out in a 17.6 ml reaction mix in which the following were present:

- 0.5 mg of A7/9 amplicon
- 0.25 mg of A8/10 amplicon

The 3' ends were filled in (30 min at 30°C) in a 20 ml reaction mix in which the following were present:

- 17.6 ml of gA7/9 and A8/10 annealing reaction (produced as described above)
- 50 mM dNTPs
- 2 ml of 1× Klenow buffer
- 2 U of Klenow enzyme

The nucleic acid coding for the modified promoter version AP3P was amplified by means of PCR using a sense-specific primer (PR7 SEQ ID NO: 33) and an antisense-specific primer (PR10 SEQ ID NO: 36).

The PCR conditions were as follows:

The PCR for amplification of the AP3P fragment was carried out in a 50 ml reaction mix in which the following were present:

- 1 ml of annealing reaction (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 33)
- 0.2 mM PR10 (SEQ ID NO: 36)
- 5 ml of 10× PCR buffer (Stratagene)
- 0.25 ml of Pfu Taq polymerase (Stratagene)
- 28.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The PCR amplification with SEQ ID NO: 33 and SEQ ID NO: 36 resulted in a 778 bp fragment which codes for the modified promoter version AP3P. The amplicon was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing with the primers T7 and M13 confirmed a sequence identical to the sequence AL132971, region 10 200 to 9298, with the internal region 9285 to 9526 having been deleted. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

The cloning was performed by isolating the 771 bp SacI-HindIII fragment from pTAP3P and ligation into the SacI-HindIII-cut vector pJIT117. The clone which comprises the promoter AP3P instead of the original promoter d35S is called pJAP3P.

For the production of an expression cassette pJAP3PKETO2, the 1027 bp SpHI fragment KETO2 was cloned into the SpHI-cut vector pJAP3P. The clone which comprises the fragment KETO2 in the correct orientation as N-terminal fusion with the rbcS transit peptide is called pJAP3PKETO2.

For the production of an expression cassette pJAP3PKETO4, the 1032 bp SpHI-EcoRI fragment KETO4 (described in Example 3) was cloned into the SpHI-EcoRI-cut vector pJAP3P. The clone which comprises the fragment KETO4 in the correct orientation as N-terminal fusion with the rbcS transit peptide is called pJAP3PKETO4.

The preparation of an expression vector for the Agrobacterium-mediated transformation of the AP3P-controlled ketolase from *Haematococcus pluvialis* in *Tagetes erecta* was carried out using the binary vector pSUN5 (WO 02/00900).

For production of the expression vector pS5AP3PKETO2, the 2.8 KB bp SacI-XhoI fragment from pJAP3PKETO2 was ligated to the SacI-XhoI-cut vector pSUN5 (Figure 4, construct map). In Figure 4, fragment *AP3P* comprises the modified AP3P

promoter (771 bp), fragment *rbcS* the rbcS transit peptide from pea (204 bp), fragment *KETO2* (1027 bp) the entire primary sequence coding for the *Haematococcus pluvialis* ketolase, fragment *term* (761 bp) the polyadenylation signal of CaMV.

Example I.5.B:

Production of expression vectors for the constitutive expression of *Nostoc sp. PCC 7120* ketolase in *Tagetes erecta*

The ketolase from *Nostoc* in *Tagetes erecta* was expressed under the control of the constitutive promoter FNR (ferredoxin-NADPH oxidoreductase) from *Arabidopsis thaliana*. The expression was carried out using the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715).

The DNA fragment which comprises the FNR promoter region -635 to -1 from *Arabidopsis thaliana* was produced by means of PCR using genomic DNA (isolated from *Arabidopsis thaliana* using standard methods) and also the primer FNR-1 (SEQ ID No. 90) and FNR-2 (SEQ ID No. 91).

The PCR conditions were as follows:

The PCR for amplification of the DNA which comprises the FNR promoter fragment FNR1-2 (-635 to -1) was carried out in a 50 µl reaction mix in which the following were present:

- 100 ng of genomic DNA from *A.thaliana*
- 0.25 mM dNTPs
- 0.2 mM FNR-1 (SEQ ID No. 90)
- 0.2 mM FNR-2 (SEQ ID No. 91)
- 5 µl of 10× PCR buffer (Stratagene)
- 0.25 µl of Pfu polymerase (Stratagene)
- 28.8 µl of distilled water.

The PCR was carried out under the following cycle conditions:

1× 94°C 2 minutes

35× 94°C 1 minute
50°C 1 minute
72°C 1 minute
1× 72°C 10 minutes

The 653 bp amplicon was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods and the plasmid pFNR was obtained.

Sequencing the clone pFNR confirmed a sequence which agreed with a sequence section of chromosome 5 of Arabidopsis thaliana (database entry AB011474) from position 70 127 to 69 493. The gene starts at base pair 69 492 and is annotated "ferredoxin-$NADP^+$ reductase".

This clone is called pFNR and was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

The cloning was performed by isolating the 635 bp SacI-HindIII fragment from pFNR and ligation into the SacI-HindIII-cut vector pJIT117. The clone which comprises the promoter FNR instead of the original promoter d35S is called pJITFNR.

For the production of an expression cassette pJFNRNOST, the 805 bp SpHI fragment NOSTF-G (described in Example 1) was cloned into the SpHI-cut vector pJITFNR. The clone which comprises the fragment NOSTF-G in the correct orientation as N-terminal fusion with the rbcS transit peptide is called pJFNRNOST.

An expression cassette for the *Agrobacterium*-mediated transformation of the expression vector with the ketolase from *Nostoc* in Tagetes erecta was produced using the binary vector pSUN5 (WO 02/00900).

For the production of the Tagetes expression vector pS5FNRNOST, the 2.4 Kb SacI-XhoI fragment (partial SacI hydrolysis) from pJFNRNOST was ligated with the SacI-XhoI-cut vector pSUN5 (Figure 5, construct map). In Figure 5, fragment *FNR promoter* comprises the duplicated FNR promoter (655 bp), fragment *rbcS Transit Peptide* the rbcS transit peptide from pea (204 bp), fragment *Nost Ketolase* (799 bp)

the entire primary sequence coding for the *Nostoc* ketolase, fragment *35S Terminator* (761 bp) comprises the polyadenylation signal of CaMV.

Example I.5C:

Production of expression vectors for the flower-specific expression of the *Nostoc sp. PCC 7120* ketolase in *Tagetes erecta*

The ketolase from *Nostoc* was expressed in Tagetes erecta using the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715). The expression was carried out under the control of a modified version AP3P of the flower-specific promoter AP3 from Arabidopsis thaliana (AL132971: nucleotide region 9298-10 200; Hill et al. (1998) Development 125: 1711-1721).

The DNA fragment which comprises the AP3 promoter region -902 to +15 from Arabidopsis thaliana was produced by means of PCR using genomic DNA (isolated from Arabidopsis thaliana using standard methods) and also the primer AP3-1 (SEQ ID No. 93) and AP3-2 (SEQ ID No. 94).

The PCR conditions were as follows:

The PCR for the amplification of the DNA which comprises the AP3 promoter fragment (-902 to +15) was carried out in a 50 µl reaction mix in which the following were present:

- 100 ng of genomic DNA from A.thaliana
- 0.25 mM dNTPs
- 0.2 mM AP3-1 (SEQ ID No. 93)
- 0.2 mM AP3-2 (SEQ ID No. 94)
- 5 µl of 10× PCR buffer (Stratagene)
- 0.25 µl of Pfu polymerase (Stratagene)
- 28.8 µl of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes

35× 94°C 1 minute
50°C 1 minute
72°C 1 minute
1× 72°C 10 minutes

The 929 bp amplicon was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods and the plasmid pAP3 was obtained.

Sequencing of the clone pAP3 confirmed a sequence which differs from the published AP3 sequence (AL132971, nucleotide region 9298-10 200) only in an insertion (a G in position 9765 of sequence AL132971) and a base exchange (a G instead of an A in position 9726 of sequence AL132971). These nucleotide differences were reproduced in an independent amplification experiment and thus represent the actual nucleotide sequence in the Arabidopsis thaliana plants used.

The modified version AP3P was produced by means of recombinant PCR using the plasmid pAP3. The region 10 200-9771 was amplified (amplicon A1/4) with the primers AP3-1 (SEQ ID No. 93) and AP3-4 (SEQ ID No. 96), the region 9526-9285 was amplified (amplicon A2/3) with the AP3-3 (SEQ ID No. 95) and AP3-2 (SEQ ID No. 94).

The PCR conditions were as follows:

The PCR reactions for amplification of the DNA fragments which comprise the regions region 10 200-9771 and region 9526-9285 of the AP3 promoter were carried out in 50 µl reaction mixes in which the following were present:

- 100 ng of AP3 amplicon (described above)
- 0.25 mM dNTPs
- 0.2 mM sense primer (AP3-1 SEQ ID No. 93 or AP3-3 SEQ ID No. 95)
- 0.2 mM antisense primer (AP3-4 SEQ ID No. 96 or AP3-2 SEQ ID No. 94)
- 5 µl of 10× PCR buffer (Stratagene)
- 0.25 µl of Pfu Taq polymerase (Stratagene)
- 28.8 µl of distilled water.

The PCR was carried out under the following cycle conditions:

1× 94°C 2 minutes
35× 94°C 1 minute
50°C 1 minute
72°C 1 minute
1× 72°C 10 minutes

The recombinant PCR comprises annealing the amplicons A1/4 and A2/3 overlapping over a sequence of 25 nucleotides, completion to give a double strand and subsequent amplification. This produces a modified version of the AP3 promoter, AP3P, in which positions 9670-9526 are deleted. The denaturation (5 min at 95°C) and annealing (slow cooling at room temperature to 40°C) of both amplicons A1/4 and A2/3 was carried out in a 17.6 µl reaction mix in which the following were present:

- 0.5 µg of A1/4 amplicon
- 0.25 µg of A2/3 amplicon

The 3' ends were filled in (30 min at 30°C) in a 20 µl reaction mix in which the following were present:

- 17.6 µl of A1/4 and A2/3 annealing reaction (produced as described above)
- 50 µM dNTPs
- 2 µl of 1× Klenow buffer
- 2 U of Klenow enzyme

The nucleic acid coding for the modified promoter version AP3P was amplified by means of PCR using a sense-specific primer (AP3-1 SEQ ID No. 93) and an antisense-specific primer (AP3-2 SEQ ID No. 94).

The PCR conditions were as follows:

The PCR for amplification of the AP3P fragment was carried out in a 50 µl reaction mix in which the following were present:

- 1 µl of annealing reaction (produced as described above)

- 0.25 mM dNTPs
- 0.2 mM AP3-1 (SEQ ID No. 93)
- 0.2 mM AP3-2 (SEQ ID No. 94)
- 5 µl of 10× PCR buffer (Stratagene)
- 0.25 µl of Pfu Taq polymerase (Stratagene)
- 28.8 µl of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The PCR amplification with SEQ ID No. 93 (AP3-1) and SEQ ID No. 94 (AP3-2) resulted in a 783 bp fragment which codes for the modified promoter version AP3P. The amplicon was cloned into the cloning vector pCR2.1 (Invitrogen) and the plasmid pAP3P was obtained. Sequencing with the primers T7 and M13 confirmed a sequence identical to the sequence AL132971, region 10 200-9298, the internal region 9285-9526 having been deleted. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

The cloning was performed by isolating the 783 bp SacI-HindIII fragment from pAP3P and ligation into the SacI-HindIII-cut vector pJIT117. The clone which comprises the promoter AP3P instead of the original promoter d35S is called pJITAP3P. For the production of an expression cassette pJAP3NOST, the 805 bp SpHI fragment NOSTF-G (described in Example 1) was cloned into the SpHI-cut vector pJITAP3P. The clone which comprises the fragment NOSTF-G in the correct orientation as N-terminal fusion with the rbcS transit peptide is called pJAP3PNOST.

An expression vector for the Agrobacterium-mediated transformation of the AP3P-controlled ketolase from Nostoc in Tagetes erecta was produced using the binary vector pSUN5 (WO02/00900).

For the production of the expression vector pS5AP3PNOST, the 2.6 KB bp SacI-XhoI (partial SacI hydrolysis) fragment from pS5AP3PNOST was ligated with the SacI-XhoI-cut vector pSUN5 (Figure 6, construct map). In Figure 6, fragment AP3P comprises the modified AP3P promoter (783 bp), fragment rbcS the rbcS transit peptide from pea (207 bp), fragment NOSTF-G (792 bp) the entire primary sequence coding for the Nostoc ketolase, fragment term (795 bp) the polyadenylation signal of CaMV.

Example I.6:
Production of transgenic Tagetes plants

Tagetes seeds are sterilized and placed on germination medium (MS medium; Murashige and Skoog, Physiol. Plant. 15 (1962), 473-497) pH 5.8, 2% sucrose). The germination takes place in a temperature/light/time interval of 18 to 28°C/20-200 mE/3 to 16 weeks, but preferably at 21°C, 20 to 70 mE, for 4 to 8 weeks.

All leaves of the plants which have developed in vitro by then are harvested and cut transversely to the middle rib. The leaf explants produced as a result having a size of from 10 to 60 mm^2 are kept in the course of the preparation in liquid MS medium at room temperature for a maximum of 2 h.

An optional Agrobacterium tumefaciens strain, but preferably a supervirulent strain, for example EHA105 having a corresponding binary plasmid which can bear a selection marker gene (preferably *bar* or *pat*) and also one or more trait or reporter genes (for example pS5KETO2 and pS5AP3PKETO2), is grown overnight and used for co-culturing with the leaf material. The growth of the bacterial strain can be performed as follows: a single colony of the corresponding strain is inoculated in YEB (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose, 0.5% magnesium sulfate·$7H_2O$) comprising 25 mg/l of kanamycin and cultured at 28°C for 16 to 20 h. The bacterial suspension is then harvested by centrifugation at 6000 g for 10 min and resuspended in liquid MS medium in such a manner that an OD_{600} of approximately 0.1 to 0.8 resulted. This suspension is used for the co-culture with the leaf material.

Immediately before the co-culture, the MS medium in which the leaves have been kept is replaced by the bacterial suspension. The leaves were incubated in the Agrobacteria suspension for 30 min with gentle shaking at room temperature. The infected explants are then placed on an agar-solidified (e.g. 0.8% plant agar (Duchefa, NL)) MS medium

comprising growth regulators, for example 3 mg/l of benzylaminopurine (BAP) and also 1 mg/l of indolylacetic acid (IAA). The orientation of the leaves on the medium is of no importance. The explants are cultured for from 1 to 8 days, but preferably for 6 days, with the following conditions being able to be used: light intensity: from 30 to 80 mmol/m^2 x sec, temperature: from 22 to 24°C, light/dark change of 16/8 hours. Then, the co-cultured explants are transferred to fresh MS medium, preferably comprising the same growth regulators, this second medium additionally comprising an antibiotic for suppressing bacterial growth. Timentin at a concentration of from 200 to 500 mg/l is very suitable for this purpose. As second selective component, one is used for selecting the success of transformation. Phosphinothricin at a concentration of from 1 to 5 mg/l selects very efficiently, but other selective components according to the method to be used are also conceivable.

After in each case from one to three weeks, the explants are transferred to fresh medium until plumules and small buds develop which are then transferred to the same basal medium including Timentin and PPT or alternative components with growth regulators, that is to say, for example, 0.5 mg/l indolylbutyric acid (IBA) and 0.5 mg/l of gibberillic acid GA_3, for rooting. Rooted buds can be transferred to the glasshouse.

In addition to the described method, the following advantageous modifications are possible:

- Before the explants are infected with the bacteria, they can be preincubated for from 1 to 12 days, preferably from 3 to 4, on the above-described medium for the co-culture. The infection, co-culture and selective regeneration are then carried out as described above.

- The pH for the regeneration (usually 5.8) can be lowered to pH 5.2. This improves the control of the Agrobacterial growth.

- The addition of $AgNO_3$ (3-10 mg/l) to the regeneration medium improves the state of the culture, including regeneration itself.

- Components which reduce the phenol formation and are known to those skilled in the art, for example citric acid, ascorbic acid, PVP and many more, have a beneficial effect on the culture.

- For the entire method, liquid culture medium can also be used. The culture can also be incubated on commercially conventional supports which are positioned on the liquid medium.

According to the above-described transformation method, using the following expression constructs, the following lines were obtained:

With pS5KETO2, for example, the following were obtained: cs18-1 and cs18-2, with pS5AP3PKETO2, for example, the following were obtained: cs19-1, cs19-2 and cs19-3.
With pS5FNRNOST, for example, the following were obtained: ms 103-1, ms103-2, ms103-3, with pS5AP3NOST, for example, the following were obtained: ms 104-1, ms104-2, ms104-3.

Example I.8
Characterization of the transgenic plant flowers

Example I.8.1
Separation of carotenoid esters in flower leaves of transgenic plants

General working instructions:

The flower leaves of the transgenic plants are ground in a mortar in liquid nitrogen and the petal powder (about 40 mg) is extracted with 100% acetone (three times, each time 500 ml). The solvent is evaporated and the carotenoids are resuspended in from 100 to 200 ml of petroleum ether/acetone (5:1, v/v).

The carotenoids are separated according to their phobicity in concentrated form by means of thin-layer chromatography (TLC) on Silica60 F254 plates (Merck) in an organic mobile phase (petroleum ether/acetone; 5:1). Yellow (xanthophyll esters), red (ketocarotenoid esters) and orange bands (mixture of xanthophyll and ketocarotenoid esters) are scraped off on the TLC.

The silica-bound carotenoids are eluted three times with 500 ml of acetone, the solvent is evaporated and the carotenoids are separated by means of HPLC and identified.

By means of a C30 reversed-phase column, mono- and diesters of carotenoids can be differentiated. HPLC running conditions were virtually identical to a published method (Frazer et al.(2000), Plant Journal 24(4): 551-558). It is possible to identify the carotenoids on the basis of the UV-VIS spectra.

Example I.9

Enzymatic hydrolysis of carotenoid esters and identification of the carotenoids

General working instructions

Petal material (50 to 100 mg fresh weight) ground in a mortar is extracted with 100% acetone (three times with 500 ml; shake each time for about 15 minutes). The solvent is evaporated. Carotenoids are then taken up in 400 ml of acetone (absorption at 475 nm between 0.75 and 1.25) and treated in the ultrasonic bath for 5 min. The carotenoid extract is mixed with 300 ml of 50 mM Tris-HCl buffer (pH 7.0) and incubated at 37°C for 5 to 10 minutes. Thereafter, from 100 to 200 ml of cholesterol esterase are added (stock solution: 6.8 units/ml of a cholesterol esterase of Pseudomonas spec.). After from 8 to 12 hours, from 100 to 200 ml of enzyme are again added; the esters are hydrolyzed within 24 hours on incubation at 37°C. After adding 0.35 g of $Na_2SO_4 \cdot 10H_2O$ and 500 ml of petroleum ether, the mixture is mixed well and centrifuged (3 minutes; 4500 g). The petroleum ether phase is taken off and once more mixed with 0.35 g of $Na_2SO_4 \cdot 10H_2O$ (anhydrous). Centrifugation for 1 minute at 10 000 g. Petroleum ether is evaporated and free carotenoids are taken up in from 100 to 120 ml of acetone. By means of HPLC and C30 reversed-phase column, free carotenoids can be identified on the basis of retention time and UV-VIS spectra.

Example I.10:

Production of a cloning vector for producing inverted-repeat expression cassettes for the flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta*

Inverted-repeat transcripts consisting of fragments of the epsilon-cyclase in *Tagetes erecta* were expressed under the control of a modified version AP3P of the flower-

specific promoter AP3 from *Arabidopsis thaliana* (AL132971: nucleotide region 9298 to 10 200; Hill et al. (1998) Development 125: 1711 to 1721).

The inverted-repeat transcript comprises in each case one fragment in correct orientation (sense fragment) and one sequence-identical fragment in the opposite orientation (antisense fragment) which are connected to one another by a functional intron, the PIV2 intron of the ST-LH1 gene from potatoes (Vancanneyt G. et al. (1990) Mol Gen Genet 220: 245-50).

The cDNA which codes for the AP3 promoter (-902 to +15) from *Arabidopsis thaliana* was produced by means of PCR using genomic DNA (isolated from *Arabidopsis thaliana* by standard method) and the primers PR7 (SEQ ID NO: 49) and PR10 (SEQ ID NO: 52).

The PCR conditions were as follows:

The PCR for amplification of the DNA which codes for the AP3 promoter fragment (-902 to +15) was carried out in a 50 ml reaction mix in which the following were present:

- 1 ml of genomic DNA from *A.thaliana* (1:100 dilution produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 49)
- 0.2 mM PR10 (SEQ ID NO: 52)
- 5 ml of 10× PCR buffer (Stratagene)
- 0.25 ml of Pfu polymerase (Stratagene)
- 28.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The 922 bp amplicon was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods and the plasmid pTAP3 was obtained. Sequencing the clone pTAP3 confirmed a sequence which differs from the published AP3 sequence (AL132971, nucleotide region 9298 to 10 200) only by an insertion (a G in position 9765 of the sequence AL132971) and a base exchange (a G instead of an A in position 9726 of sequence AL132971) (position 33: T instead of G, position 55: T instead of G). These nucleotide differences were reproduced in an independent amplification experiment and thus represent the nucleotide sequence in the *Arabidopsis thaliana* plant used.

The modified version AP3P was produced by means of recombinant PCR using the plasmid pTAP3. The region 10 200 to 9771 was amplified with the primers PR7 (SEQ ID NO: 49) and primers PR9 (SEQ ID NO: 51) (amplicon A7/9), the region 9526 to 9285 was amplified with PR8 (SEQ ID NO: 50) and PR10 (SEQ ID NO: 52) (amplicon A8/10).

The PCR conditions were as follows:

The PCR reactions for amplification of the DNA fragments which code for the regions region 10 200 to 9771 and 9526 to 9285 of the AP3 promoter were carried out in 50 ml reaction mixes in which the following were present:

- 100 ng of AP3 amplicon (described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 49) or PR8 (SEQ ID NO: 50)
- 0.2 mM PR9 (SEQ ID NO: 51) or PR10 (SEQ ID NO: 52)
- 5 ml of 10× PCR buffer (Stratagene)
- 0.25 ml of Pfu Taq polymerase (Stratagene)
- 28.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	50°C	2 minutes

	72°C	3 minutes
1×	72°C	10 minutes

The recombinant PCR comprises annealing of the amplicons A7/9 and A8/10 which are overlapping over a sequence of 25 nucleotides, completion to form a double strand and subsequent amplification. This produces a modified version of the AP3 promoter, AP3P, in which the positions 9670 to 9526 are deleted. The denaturation (5 min at 95°C) and annealing (slow cooling at room temperature to 40°C) of both amplicons A7/9 and A8/10 was carried out in a 17.6 ml reaction mix in which the following were present:

- 0.5 mg of A7/9
- 0.25 mg of A8/10

The 3' ends were filled in (30 min at 30°C) in a 20 ml reaction mix in which the following were present:

- 17.6 ml of A7/9 and A8/10 annealing reaction (produced as described above)
- 50 mM dNTPs
- 2 ml of 1× Klenow buffer
- 2 U of Klenow enzyme

The nucleic acid coding for the modified promoter version AP3P was amplified by means of PCR using a sense-specific primer (PR7 SEQ ID NO: 49) and an antisense-specific primer (PR10 SEQ ID NO: 52).

The PCR conditions were as follows:

The PCR for the amplification of the AP3P fragment was carried out in a 50 ml reaction mix in which the following were present:

- 1 ml of annealing reaction (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 49)
- 0.2 mM PR10 (SEQ ID NO: 52)
- 5 ml of 10× PCR buffer (Stratagene)

- 0.25 ml of Pfu Taq polymerase (Stratagene)
- 28.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The PCR amplification with PR7, SEQ ID NO: 49 and PR10 SEQ ID NO: 52 resulted in a 778 bp fragment which codes for the modified promoter version AP3P. The amplicon was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing with the primers T7 and M13 confirmed a sequence identical to the sequence AL132971, region 10 200 to 9298, with the internal region 9285 to 9526 having been deleted. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

The cloning was carried out by isolating the 771 bp SacI-HindIII fragment from pTAP3P and ligation into the SacI-HindIII-cut vector pJIT117. The clone which comprises the promoter AP3P instead of the original promoter d35S is called pJAP3P.

A DNA fragment which comprises the PIV2 intron of the gene ST-LS1 was amplified by means of PCR using plasmid DNA p35SGUS INT (Vancanneyt G. et al. (1990) Mol Gen Genet 220: 245-50) and also the primers PR40 (Seq ID NO: 54) and PR41 (Seq ID NO: 55).

The PCR conditions were as follows:

The PCR for amplification of the sequence of the intron PIV2 of the gene ST-LS1 was carried out in a 50 ml reaction mix in which the following were present:

- 1 ml of p35SGUS INT
- 0.25 mM dNTPs
- 0.2 mM PR40 (SEQ ID NO: 54)

- 0.2 mM PR41 (SEQ ID NO: 55)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 28.8 ml of distilled water.

The PCR was carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The PCR amplification using PR40 and PR41 resulted in a 206 bp fragment. Using standard methods, the amplicon was cloned into the PCR cloning vector pBluntII (Invitrogen) and the clone pBluntII-40-41 was obtained. Sequencing this clone with the primer SP6 confirmed a sequence which is identical to the corresponding sequence from the vector p35SGUS INT.

This clone was therefore used for cloning into the vector pJAP3P (described above).

The cloning was performed by isolating the 206 bp SalI-BamHI fragment from pBluntII-40-41 and ligation to the SalI-BamHI-cut vector pJAP3P. The clone which comprises the intron PIV2 of the gene ST-LS1 in the correct orientation then to the 3' end of the rbcs transit peptide is called pJAI1 and is suitable for producing expression cassettes for the flower-specific expression of inverted-repeat transcripts.

In Figure 7, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *rbcs* the rbcS transit peptide from pea (204 bp), fragment *intron* the intron PIV2 of the potato gene ST-LS1, and fragment *term* (761 bp) the polyadenylation signal of CaMV.

Example I.11

Production of inverted-repeat expression cassettes for the flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta* (directed against the 5' region of the epsilon-cyclase cDNA)

The nucleic acid which comprises the 5'-terminal 435 bp region of the epsilon-cyclase cDNA (Genbank accession NO: AF251016) was amplified by means of the polymerase chain reaction (PCR) from *Tagetes erecta* cDNA using a sense-specific primer (PR42 SEQ ID NO: 56) and an antisense-specific primer (PR43 SEQ ID NO: 57). The 5'-terminal 435 bp region of the epsilon-cyclase cDNA from *Tagetes erecta* is composed of 138 bp 5'-non-translated sequence (5'UTR) and 297 bp of the coding region corresponding to the N terminus.

For the preparation of total RNA from flowers of Tagetes, 100 mg of the frozen pulverized flowers were transferred to a reaction vessel and taken up in 0.8 ml of Trizol buffer (LifeTechnologies). The suspension was extracted with 0.2 ml of chloroform. After centrifugation at 12 000 g for 15 minutes, the aqueous supernatant was taken off and transferred to a new reaction vessel and extracted with one volume of ethanol. The RNA was precipitated with one volume of isopropanol, washed with 75% ethanol and the pellet was dissolved in DEPC water (overnight incubation of water with 1/1000 volume of diethyl pyrocarbonate at room temperature, then autoclaved). The RNA concentration was determined photometrically. For the cDNA synthesis, 2.5 μg of total RNA were denatured at 60°C for 10 min, cooled on ice for 2 min and transcribed into cDNA by means of a cDNA kit (Ready-to-go-you-prime-beads, Pharmacia Biotech) according to manufacturer's instructions, using an antisense-specific primer (PR17 SEQ ID NO: 53).

The conditions of the subsequent PCR reactions were as follows:

The PCR for the amplification of the PR42-PR43 DNA fragment which comprises the 5'-terminal 435 bp region of the epsilon-cyclase was performed in a 50 ml reaction mix in which the following were present:

- 1 ml of cDNA (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR42 (SEQ ID NO: 56)
- 0.2 mM PR43 (SEQ ID NO: 57)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 28.8 ml of distilled water.

The PCR for the amplification of the PR44-PR45 DNA fragment which comprises the 5'-terminal 435 bp region of the epsilon-cyclase was performed in a 50 ml reaction mix in which the following were present:

- 1 ml of cDNA (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR44 (SEQ ID NO: 58)
- 0.2 mM PR45 (SEQ ID NO: 59)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 28.8 ml of distilled water.

The PCR reactions were carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	58°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The PCR amplification using primer PR42 and PR43 resulted in a 443 bp fragment, and the PCR amplification using primer PR44 and PR45 resulted in a 444 bp fragment.

The two amplicons, the PR42-PR43 (HindIII-SalI sense) fragment and the PR44-PR45 (EcoRI-BamHI antisense) fragment, were cloned using standard methods into the PCR cloning vector pCR-BluntII (Invitrogen). Sequencing with the primer SP6 confirmed in each case a sequence identical to the published sequence AF251016 (SEQ ID NO: 38), apart from the introduced restriction sites. This clone was therefore used for the production of an inverted-repeat construct in the cloning vector pJAI1 (see Example I.10).

The first cloning step was performed by isolating the 444 bp PR44-PR45 BamHI-EcoRI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation to the BamHI-EcoRI-cut vector pJAI1. The clone which comprises the 5'-terminal region of

the epsilon-cyclase in the antisense orientation is called pJAI2. The ligation produces a transcriptional fusion between the antisense fragment of the 5'-terminal region of the epsilon-cyclase and the polyadenylation signal from CaMV.

The second cloning step was performed by isolating the 443 bp PR42-PR43 HindIII-SalI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation to the HindIII-SalI-cut vector pJAI2. The clone which comprises the 435 bp 5'-terminal region of the epsilon-cyclase cDNA in the sense orientation is called pJAI3. The ligation produces a transcriptional fusion between the AP3P and the sense fragment of the 5' terminal region of the epsilon-cyclase.

For the production of an inverted-repeat expression cassette under the control of the CHRC promoter, a CHRC promoter fragment was amplified using genomic DNA from petunia (produced according to standard methods) and also the primers PRCHRC5 (SEQ ID NO: 76) and PRCHRC3 (SEQ ID NO: 77). The amplicon was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing the resulting clone pCR2.1-CHRC using the primers M13 and T7 confirmed a sequence identical to the sequence AF099501. This clone was therefore used for cloning into the expression vector pJAI3.

The cloning was carried out by isolating the 1537 bp SacI-HindIII fragment from pCR2.1-CHRC and ligation into the SacI-HindIII-cut vector pJAI3. The clone which comprises the promoter CHRC instead of the original promoter AP3P is called pJCI3.

The expression vectors for the Agrobacterium-mediated transformation of the AP3P- or CHRC-controlled inverted-repeat transcript in *Tagetes erecta* were produced using the binary vector pSUN5 (WO02/00900).

For production of the expression vector pS5AI3, the 2622 bp SacI-XhoI fragment from pJAI3 was ligated to the SacI-XhoI-cut vector pSUN5 (Figure 8, construct map).

In Figure 8, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *5sense* the 5' region of the epsilon-cyclase from *Tagetes erecta* (435 bp) in the sense orientation, fragment *intron* the intron PIV2 of the potato gene ST-LS1, fragment *5anti* the 5' region of the epsilon-cyclase from *Tagetes erecta* (435 bp) in the antisense orientation, and fragment *term* (761 bp) the polyadenylation signal of CaMV.

For the production of the expression vector pS5CI3, the 3394 bp SacI-XhoI fragment from pJCI3 was ligated to the SacI-XhoI-cut vector pSUN5 (Figure 9, construct map).

In Figure 9, fragment *CHRC* comprises the promoter (1537 bp), fragment *5sense* the 5' region of the epsilon-cyclase from *Tagetes erecta* (435 bp) in the sense orientation, fragment *intron* the intron PIV2 of the potato gene ST-LS1, fragment *5anti* the 5' region of the epsilon-cyclase from *Tagetes erecta* (435 bp) in the antisense orientation, and fragment *term* (761 bp) the polyadenylation signal of CaMV.

Example I.12

Production of an inverted-repeat expression cassette for the flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta* (directed against the 3' region of the epsilon-cyclase cDNA)

The nucleic acid which comprises the 3'-terminal region (384 bp) of the epsilon-cyclase cDNA (Genbank accession NO: AF251016) was amplified by means of the polymerase chain reaction (PCR) from *Tagetes erecta* cDNA using a sense-specific primer (PR46 SEQ ID NO: 60) and an antisense-specific primer (PR47 SEQ ID NO: 61). The 3'-terminal region (384 bp) of the epsilon-cyclase cDNA from *Tagetes erecta* is composed of a 140 bp 3'-non-translated sequence (3'UTR) and 244 bp of the coding region corresponding to the C terminus.

Total RNA was prepared from flowers of Tagetes as described under Example I.11.

The cDNA was synthesized as described under Example I.11, using the antisense-specific primer PR17 (SEQ ID NO: 53).

The conditions for the subsequent PCR reactions were as follows:

The PCR for amplification of the PR46-PR47 DNA fragment which comprises the 3'-terminal 384 bp region of the epsilon-cyclase was performed in a 50 ml reaction mix in which the following were present:

- 1 ml of cDNA (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR46 (SEQ ID NO: 60)

- 0.2 mM PR47 (SEQ ID NO: 61)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 28.8 ml of distilled water.

The PCR for amplification of the PR48-PR49 DNA fragment which comprises the 5'-terminal 384 bp region of the epsilon-cyclase was performed in a 50 ml reaction mix in which the following were present:

- 1 ml of cDNA (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR48 (SEQ ID NO: 62)
- 0.2 mM PR49 (SEQ ID NO: 63)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 28.8 ml of distilled water.

The PCR reactions were carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	58°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The PCR amplification using SEQ ID NO: 60 and SEQ ID NO: 61 resulted in a 392 bp fragment, and the PCR amplification using SEQ ID NO: 62 and SEQ ID NO: 63 resulted in a 396 bp fragment.

The two amplicons, the PR46-PR47 fragment and the PR48-PR49 fragment, were cloned using standard methods into the PCR cloning vector pCR-BluntII (Invitrogen). Sequencing using the primer SP6 confirmed in each case a sequence identical to the published sequence AF251016 (SEQ ID NO: 38) apart from the restriction sites introduced. This clone was therefore used for production of an inverted-repeat construct in the cloning vector pJAI1 (see Example I.10).

The first cloning step was performed by isolating the 396 bp PR48-PR49 BamHI-EcoRI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation to the BamHI-EcoRI-cut vector pJAI1. The clone which comprises the 3'-terminal region of the epsilon-cyclase in the antisense orientation is called pJAI4. The ligation produces a transcriptional fusion between the antisense fragment of the 3'-terminal region of the epsilon-cyclase and the polyadenylation signal from CaMV.

The second cloning step was performed by isolating the 392 bp PR46-PR47 HindIII-SalI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation to the HindIII-SalI-cut vector pJAI4. The clone which comprises the 392 bp 3'-terminal region of the epsilon-cyclase cDNA in the sense orientation is called pJAI5. The ligation produces a transcriptional fusion between the AP3P and the sense fragment 3'-terminal region of the epsilon-cyclase.

An expression vector for the Agrobacterium-mediated transformation of the AP3P-controlled inverted-repeat transcript in Tagetes erecta was produced using the binary vector pSUN5 (WO02/00900). For production of the expression vector pS5AI5, the 2523 bp SacI-XhoI fragment from pJAI5 was ligated to the SacI-XhoI-cut vector pSUN5 (Figure 10, construct map).

In Figure 10, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *3sense* the 3' region of the epsilon-cyclase from Tagetes erecta (435 bp) in the sense orientation, fragment *intron* the intron IV2 of the potato gene ST-LS1, fragment *3anti* the 3' region of the epsilon-cyclase from Tagetes erecta (435 bp) in the antisense orientation, and fragment *term* (761 bp) the polyadenylation signal of CaMV.

Example I.13
Cloning the epsilon-cyclase promoter

A 199 bp fragment and the 312 bp fragment of the epsilon-cyclase promoter were isolated by two independent cloning strategies, inverse PCR (adapted from Long et al. Proc. Natl. Acad. Sci USA 90: 10370) and TAIL-PCR (Liu Y-G. et al. (1995) Plant J. 8: 457-463) using genomic DNA (isolated by standard method from *Tagetes erecta*, Orangenprinz line).

For the inverse PCR approach, 2 µg of genomic DNA were digested in a 25 µl reaction mix with EcoRV and RsaI, then diluted to 300 ml and religated overnight at 16°C using 3 U of ligase. Using the primers PR50 (SEQ ID NO: 64) and PR51 (SEQ ID NO: 65), by PCR amplification, a fragment was produced which, in each case in the sense orientation, comprises 354 bp of the epsilon-cyclase cDNA (Genbank Accession AF251016), ligated to 300 bp of the epsilon-cyclase promoter and also 70 bp of the 5'-terminal region of the cDNA epsilon-cyclase (see Figure 11).

The conditions of the PCR reactions were as follows:

The PCR for amplification of the PR50-PR51 DNA fragment which, inter alia, comprises the 312 bp promoter fragment of the epsilon-cyclase was performed in a 50 ml reaction mix in which the following were present:

- 1 ml of ligation mix (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR50 (SEQ ID NO: 64)
- 0.2 mM PR51 (SEQ ID NO: 65)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 28.8 ml of distilled water.

The PCR reactions were carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The PCR amplification using primer PR50 and PR51 resulted in a 734 bp fragment which, inter alia, comprises the 312 bp promoter fragment of epsilon-cyclase (Figure 11).

The amplicon was cloned using standard methods into the PCR cloning vector pCR2.1 (Invitrogen). Sequencing using the primers M13 and T7 gave the sequence SEQ ID NO: 45. This sequence was reproduced in an independent amplification experiment and thus represents the nucleotide sequence in the *Tagetes erecta* line Orangenprinz used.

For the TAIL-PCR approach, three successive PCR reactions were carried out using in each case different gene-specific primers (nested primers).

The TAIL1-PCR was performed in a 20 ml reaction mix in which the following were present:

- 1 ng of genomic DNA (produced as described above)
- 0.2 mM each dNTP
- 0.2 mM PR60 (SEQ ID NO: 66)
- 0.2 mM AD1 (SEQ ID NO: 69)
- 2 ml of 10× PCR buffer (TAKARA)
- 0.5 U of R Taq polymerase (TAKARA)
- made up to 20 ml with distilled water.

- AD1 was first a mixture of primers of the sequences (a/c/g/t)tcga(g/c)t(a/t)t(g/c)g(a/t)gtt.

The PCR reaction TAIL1 was carried out under the following cycle conditions:

1× 93°C: 1 minute, 95°C: 1 minute
5× 94°C: 30 seconds, 62°C: 1 minute, 72°C: 2.5 minutes
1× 94°C: 30 seconds, 25°C: 3 minutes, ramp to 72°C in 3 minutes, 72°C: 2.5 minutes
15× 94°C: 10 seconds, 68°C: 1 minute, 72°C: 2.5 minutes; 94°C: 10 seconds, 68°C: 1 minute, 72°C: 2.5 minutes; 94°C: 10 seconds, 29°C: 1 minute, 72°C: 2.5 minutes
1× 72°C: 5 minutes

The TAIL2-PCR was performed in a 21 ml reaction mix in which the following were present:

- 1 ml of a 1:50 dilution of the TAIL1 reaction mix (produced as described above)
- 0.8 mM dNTP
- 0.2 mM PR61 (SEQ ID NO: 67)
- 0.2 mM AD1 (SEQ ID NO: 69)
- 2 ml of 10× PCR buffer (TAKARA)
- 0.5 U of R Taq polymerase (TAKARA)
- made up to 21 ml with distilled water.

The PCR reaction TAIL2 was carried out under the following cycle conditions:

12× 94°C: 10 seconds, 64°C: 1 minute, 72°C: 2.5 minutes;
94°C: 10 seconds, 64°C: 1 minute, 72°C: 2.5 minutes;
94°C: 10 seconds, 29°C: 1 minute, 72°C: 2.5 minutes
1× 72°C: 5 minutes

The TAIL3-PCR was performed in a 100 ml reaction mix in which the following were present:

- 1 ml of a 1:10 dilution of the TAIL2 reaction mix (produced as described above)
- 0.8 mM dNTP
- 0.2 mM PR63 (SEQ ID NO: 68)
- 0.2 mM AD1 (SEQ ID NO: 69)
- 10 ml of 10× PCR buffer (TAKARA)
- 0.5 U of R Taq polymerase (TAKARA)
- made up to 100 ml with distilled water.

The PCR reaction TAIL3 was carried out under the following cycle conditions:

20× 94°C: 15 seconds, 29°C: 30 seconds, 72°C: 2 minutes
1× 72°C: 5 minutes

The PCR amplification using primer PR63 and AD1 resulted in a 280 bp fragment which, inter alia, comprises the 199 bp promoter fragment of epsilon-cyclase (Figure 12).

The amplicon was cloned using standard methods into the PCR cloning vector pCR2.1 (Invitrogen). Sequencing using the primers M13 and T7 gave the sequence SEQ ID NO: 46. This sequence is identical to the sequence SEQ ID NO: 45 which was isolated by the IPCR strategy and thus represents the nucleotide sequence in the *Tagetes erecta* line Orangenprinz used.

The pCR2.1 clone which comprises the 312 bp fragment (SEQ ID NO: 45) of the epsilon-cyclase promoter which was isolated by the IPCR strategy is called pTA-ecycP and was used for producing the IR constructs.

Example I.14

Production of an inverted-repeat expression cassette for the flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta* (directed against the promoter region of the epsilon-cyclase cDNA)

The expression of inverted-repeat transcripts consisting of promoter fragments of the epsilon-cyclase in *Tagetes erecta* was performed under the control of a modified version AP3P of the flower-specific promoter AP3 from *Arabidopsis* (see Example I.10) or of the flower-specific promoter CHRC (Genbank accession NO: AF099501). The inverted-repeat transcript comprises in each case one epsilon-cyclase promoter fragment in correct orientation (sense fragment) and a sequence-identical epsilon-cyclase promoter fragment in the opposite orientation (antisense fragment) which are joined together by a functional intron (see Example I.10).

The promoter fragments were produced by means of PCR using plasmid DNA (clone pTA-ecycP, see Example I.13) and the primers PR124 (SEQ ID NO: 70) and PR126 (SEQ ID NO: 72) and the primers PR125 (SEQ ID NO: 71) and PR127 (SEQ ID NO: 73).

The conditions of the PCR reactions were as follows:

The PCR for amplification of the PR124-PR126 DNA fragment which comprises the promoter fragment of the epsilon-cyclase was performed in a 50 ml reaction mix in which the following were present:

- 1 ml of cDNA (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR124 (SEQ ID NO: 70)
- 0.2 mM PR126 (SEQ ID NO: 72)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 28.8 ml of distilled water.

The PCR for amplification of the PR125-PR127 DNA fragment which comprises the 312 bp promoter fragment of the epsilon-cyclase was performed in a 50 ml reaction mix in which the following were present:

- 1 ml of cDNA (produced as described above)
- 0.25 mM dNTPs
- 0.2 mM PR125 (SEQ ID NO: 71)
- 0.2 mM PR127 (SEQ ID NO: 73)
- 5 ml of 10× PCR buffer (TAKARA)
- 0.25 ml of R Taq polymerase (TAKARA)
- 28.8 ml of distilled water.

The PCR reactions were carried out under the following cycle conditions:

1×	94°C	2 minutes
35×	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1×	72°C	10 minutes

The PCR amplification using primer PR124 and PR126 resulted in a 358 bp fragment, and the PCR amplification using primer PR125 and PR127 resulted in a 361 bp fragment.

The two amplicons, the PR124-PR126 (HindIII-SalI sense) fragment and the PR125-PR127 (EcoRI-BamHI antisense) fragment, were cloned using standard methods into the PCR cloning vector pCR-BluntII (Invitrogen). Sequencing using the primer SP6 confirmed in each case a sequence which, apart from the restriction sites introduced, is identical to SEQ ID NO: 45. This clone was therefore used for the production of an inverted-repeat construct in the cloning vector pJAI1 (see Example I.10).

The first cloning step was performed by isolating the 358 bp PR124-PR126 HindIII-SalI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation to the BamHI-EcoRI-cut vector pJAI1. The clone which comprises the epsilon-cyclase promoter fragment in the sense orientation is called cs43. The ligation introduces the sense fragment of the epsilon-cyclase promoter between the AP3P promoter and the intron.

The second cloning step was performed by isolating the 361 bp PR125-PR127 BamHI-EcoRI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation to the BamHI-EcoRI-cut vector cs43. The clone which comprises the epsilon-cyclase promoter fragment in the antisense orientation is called cs44. The ligation forms a transcriptional fusion between the intron and the antisense fragment of the epsilon-cyclase promoter.

For the production of an inverted-repeat expression cassette under the control of the CHRC promoter, a CHRC promoter fragment was amplified using genomic DNA from petunia (produced according to standard methods) and also the primer PRCHRC3' (SEQ ID NO: 77) and PRCHRC5' (SEQ ID NO: 76). The amplicon was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing of the resultant clone pCR2.1-CHRC using the primers M13 and T7 confirmed a sequence identical to the sequence AF099501. This clone was therefore used for cloning into the expression vector cs44.

The cloning was performed by isolating the 1537 bp SacI-HindIII fragment from pCR2.1-CHRC and ligation into the SacI-HindIII-cut vector cs44. The clone which comprises the promoter CHRC instead of the original promoter AP3P is called cs45.

For the production of an inverted-repeat expression cassette under the control of two promoters, the CHRC promoter and the AP3P promoter, the AP3P promoter was cloned into cs45 in antisense orientation to the 3' terminus of the epsilon-cyclase antisense fragment. The AP3P promoter fragment from pJAI1 was amplified using the primers PR128 and PR129. The amplicon was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing using the primers M13 and T7 confirmed a sequence identical to the sequence SEQ ID NO: 28 (AL132971). This clone pCR2.1-AP3PSX was used for the production of an inverted-repeat expression cassette under the control of two promoters.

The cloning was performed by isolating the 771 bp SalI-XhoI fragment from pCR2.1-AP3PSX and ligation into the XhoI-cut vector cs45. The clone which comprises on the 3' side of the inverted repeat the promoter AP3P in antisense orientation is called cs46.

The expression vectors for the Agrobacterium-mediated transformation of the AP3P-controlled inverted-repeat transcript in Tagetes erecta were produced using the binary vector pSUN5 (WO 02/00900).

For the production of the expression vector pS5AI7, the 1685 bp SacI-XhoI fragment from cs44 was ligated to the SacI-XhoI-cut vector pSUN5 (Figure 13, construct map). In Figure 13, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *P-sense* the 312 bp promoter fragment of the epsilon-cyclase in sense orientation, fragment *intron* the intron IV2 of the potato gene ST-LS1), and fragment *P-anti* the 312 bp promoter fragment of the epsilon-cyclase in antisense orientation.

For the production of the expression vector pS5CI7, the 2445 bp SacI-XhoI fragment from cs45 was ligated to the SacI-XhoI-cut vector pSUN5 (Figure 14, construct map).

In Figure 14, fragment *CHRC* comprises the CHRC promoter (1537 bp), fragment *P-sense* the 312 bp promoter fragment of the epsilon-cyclase in sense orientation, fragment *intron* the intron IV2 of the potato gene ST-LS1), and fragment *P-anti* the 312 bp promoter fragment of the epsilon-cyclase in antisense orientation.

For the production of the expression vector pS5CAI7, the 3219 bp SacI-XhoI fragment from cs46 was ligated to the SacI-XhoI-cut vector pSUN5 (Figure 15, construct map).

In Figure 15, fragment *CHRC* comprises the CHRC promoter (1537 bp), fragment *P-sense* the 312 bp promoter fragment of the epsilon-cyclase in sense orientation, fragment *intron* the intron IV2 of the potato gene ST-LS1), fragment *P-anti* the 312 bp promoter fragment of the epsilon-cyclase in antisense orientation and the fragment *AP3P* the 771 bp AP3P promoter fragment in antisense orientation.

Example I.15

Production of transgenic Tagetes plants having reduced ε-cyclase activity

Tagetes seeds are sterilized and placed on germination medium (MS medium; Murashige and Skoog, Physiol. Plant. 15 (1962), 473-497) pH 5.8, 2% sucrose). The germination takes place in a temperature/light/time interval of from 18 to 28°C/from 20 to 200 mE/from 3 to 16 weeks, but preferably at 21°C, from 20 to 70 mE, for from 4 to 8 weeks.

All leaves of the plants which have developed in vitro by then are harvested and cut transversely to the middle rib. The resulting leaf explants having a size of from 10 to 60 mm^2 are stored in liquid MS medium at room temperature for a maximum of 2 h in the course of the preparation.

The Agrobacterium tumefaciens strain EHA105 was transformed using the binary plasmid pS5AI3. The transformed A. tumefaciens strain EHA105 was grown overnight under the following conditions: an individual colony was inoculated into YEB (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose, 0.5% magnesium sulfate·.$7H_2O$) comprising 25 mg/l of kanamycin and grown at 28°C for from 16 to 20 h. The bacterial suspension was then harvested by centrifugation at 6000 g for 10 min and resuspended in liquid MS medium in such a manner that an OD_{600} of approximately from 0.1 to 0.8 resulted. This suspension was used for the co-culture with the leaf material.

Immediately before the co-culture, the MS medium in which the leaves had been kept is replaced by the bacterial suspension. The leaves were incubated in the Agrobacteria suspension for 30 min with gentle shaking at room temperature. The infected explants are then placed on an agar-solidified (for example 0.8% plant agar (Duchefa, NL)) MS medium comprising growth regulators, for example 3 mg/l of benzylaminopurine (BAP)

and also 1 mg/l of indolylacetic acid (IAA). The orientation of the leaves on the medium is of no importance. The explants are cultured for from 1 to 8 days, but preferably for 6 days, the following conditions being able to be used: light intensity: from 30 to 80 mmol/m^2 x sec, temperature: from 22 to 24°C, light/dark change of 16/8 hours. The co-cultured explants are then transferred to fresh MS medium, preferably comprising the same growth regulators, this second medium additionally comprising an antibiotic for suppressing bacterial growth. Timentin at a concentration of from 200 to 500 mg/l is very suitable for this purpose. As second selective component, use is made of a component for selecting the transformation success. Phosphinothricin at a concentration of from 1 to 5 mg/l selects very efficiently, but other selective components are also conceivable, according to the method to be used.

After in each case from one to three weeks, the explants are transferred to fresh medium until plumules and small buds develop which are then transferred to the same basal medium including Timentin and PPT or alternative components comprising growth regulators, that is to say, for example, 0.5 mg/l of indolylbutyric acid (IBA) and 0.5 mg/l of gibberillic acid GA_3, for rooting. Rooted buds can be transferred to the glasshouse.

In addition to the described method, the following advantageous modifications are possible:

- Before the explants are infected with the bacteria, they can be preincubated for from 1 to 12 days, preferably from 3 to 4, on the above-described medium for the co-culture. Then the infection, co-culture and selective regeneration are performed as described above.

- The pH for the regeneration (usually 5.8) can be lowered to pH 5.2. This improves the control of the Agrobacterial growth.

- The addition of $AgNO_3$ (3-10 mg/l) to the regeneration medium improves the state of the culture, including regeneration itself.

- Components which reduce the phenol formation and are known to those skilled in the art, for example citric acid, ascorbic acid, PVP, and many others, have a beneficial effect on the culture.

- For the entire method, liquid culture medium can also be used. The culture can also be incubated on commercially conventional supports which are positioned on the liquid medium.

According to the above-described transformation method, using the expression construct pS5AI3, the following lines were obtained:

CS30-1, CS30-3 and CS30-4

Example I.16:
Characterization of the transgenic Tagetes plants having reduced ε-cyclase activity

The flower material of the transgenic Tagetes erecta plants from Example I.15 was ground in a mortar in liquid nitrogen and the powder (from about 250 to 500 mg) was extracted with 100% acetone (three times, each 500 ml). The solvent was evaporated and the carotenoids were resuspended in 100 ml of acetone.

By means of a C30 reversed-phase column, the individual carotenoids were quantified. The HPLC running conditions were virtually identical to a published method (Frazer et al. (2000), Plant Journal 24(4): 551-558). It was possible to identify the carotenoids on the basis of the UV-VIS spectra.

Table 2 shows the carotenoid profile in Tagetes petals of the transgenic Tagetes plants and control Tagetes plants produced in accordance with the above-described examples. All carotenoid quantities are given in [µg/g] fresh weight, percentage changes compared with the control plant are given in brackets.

Compared with the non-genetically modified control plant, the genetically modified plants having reduced epsilon-cyclase activity have a significantly increased content of carotenoids of the "β-carotenepath", for example β-carotene and zeaxanthin and a markedly reduced content of carotenoids of the "α–carotene path", for example lutein.

Table 2

Plant	Lutein	β-Carotene	Zeaxanthin	Violaxanthin	Total carotenoids
Control	260	4.8	2.7	36	304
CS 30-1	35 (-86%)	13 (+170%)	4.4 (+62%)	59 (+63%)	111 (-63%)
Control	456	6.4	6.9	58	527
CS 30-3	62 (-86%)	13 (+103%)	8.9 (+29%)	75 (+29%)	159 (-70%)
CS 30-4	68 (-85%)	9.1 (+42%)	5.7 (-17%)	61 (+5%)	144 (-73%)

Example II

Production of astaxanthin-containing parts of plants of the genus Tagetes

The flower heads or the petals of the astaxanthin-containing plants of the genus Tagetes produced according to Example I.6 are separated off and dried. The dried flower heads or petals are then converted to powder form by comminution.

Example III

Production of astaxanthin-containing extracts and further purification

Dried flower leaves or dried flower heads of Tagetes erecta, produced in accordance with Example I.6 are homogenized in a homogenizer with an excess (about 10 parts of solvent with one part of plant material) of solvent (for example acetone, hexane, methylene chloride, methyl tertiary-butyl ether, tetrahydrofuran, ethanol, heptane, cycloheptane or petroleum ether, but not restricted exclusively to these) or with a solvent mixture (for example acetone/hexane, ethanol/hexane (50:50, v/v) or acetone/methanol (7:3, v/v) and extracted with shaking in the dark and in the cold. The residue can be re-extracted up to three times with the solvent/solvent mixture used. The collected organic solvent or solvent mixture is evaporated using an evaporator until a reduced concentrate is obtained. In addition, the material can further be extracted with hexane. The hexane used is (again in the dark and in the cold) evaporated.

The concentrate produced in this way is dissolved in hexane and chromatographed by means of column chromatography using silica material. One part of silica material for this is mixed with 1-2 parts of carotenoid solution and packed into a column. The

column is extensively washed with hexane in the dark and in the cold. The eluate is discarded. Ketocarotenoids, particularly astaxanthin, are eluted by a mixture of hexane and ethanol (2-5% ethanol in hexane) until an orange-reddish fraction elutes. This orange-reddish eluate is collected until the color changes. The orange-reddish eluate comprises astaxanthin as a mixture of mono- and diesters.

Example IV

Production of extruded trout feed, comprising astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes

The following components are extruded in a double-screw extruder.

		Weight for 500 kg
Components	***(%)***	***kg***
Fish meal	30.00	150.00
Full fat soybeans	20.00	100.00
Pregelatinized wheat starch	18.00	90.00
Vitamin premix	0.80	4.00
Choline chloride (50%)	0.20	1.00
Wheat gluten	20.00	100.00
Sipernat 50S	3.00	15.00
Fish oil	8.00	40.00

The pulverulent processed astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes, produced for example according to Example II are added as component before the extrusion.

The astaxanthin-containing extracts or processed extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes are sprayed in liquid form, for example

produced according to Example III, onto the extrudate after the extrusion (application by PPA method).

The dosage rate of astaxanthin active compound is 10, 20 and 40 mg of astaxanthin per kg of diet.

After completion of the extrusion process, the extrudate is dried and cooled.

Example V

Oral administration of astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes to trout in a standard trout feed – examination of bioavailability

The trout feed comprising the inventive astaxanthin pigments is produced in accordance with Example IV and administered orally to trout (mean live mass 180 g). 3 concentrations are tested: 10, 20 and 40 mg of astaxanthin from the inventive astaxanthin pigmenting per kg of diet.

The trout are raised as described hereinafter:

- The trout are given as standard an adaptation phase of 14 days.

- During the feeding experiment, 10 trout are kept per pool in constant-flow plastic tanks of volume 80 l of water. The water temperature is 15°C. The water is biologically purified and at least 10% of the total amount of water is replaced by fresh water per day.

- The illumination period is 12 hours per day to avoid premature sexual maturation of the animals.

- The number of pools per treatment is 3. This is equivalent to 30 trout per dose level.

- The diets are stored at -20°C to avoid astaxanthin losses. The feed is thawed by portions (weekly) and administered.

- The experimental period is 8 weeks.

Trout feeding is performed as described hereinafter:

- The experimental diets administered are extruded trout feed produced in accordance with Example IV which is additionally oil coated.

- During the adaptation phase, extruded oil-coated astaxanthin-free standard trout feed in accordance with Example IV without astaxanthin is administered.

- As negative control, extruded oil-coated astaxanthin-free standard trout feed according to Example IV without astaxanthin is administered for the entire experimental period.

- Feeding is performed 2× per day by hand until the animals are replete.

The influence of the inventive astaxanthin pigmenting not only on performance parameters of the fish, such as feed intake, feed utilization and live mass gain, but also on the bioefficiency of pigmenting is studied.

The average feed consumption per fish, feed conversion and live mass gain are statistically evaluated.

The pigmenting of the fish is measured by reflectance spectrophotometric measurements (Minolta a value = red value at the fillet incision) and by determining the astaxanthin content (mg/kg) in the fillet in each case compared with the negative control.

The Minolta a values which represent the red portion of the color tone, increase with decreasing gradient of the function in a dose-dependent manner. The Minolta b values which reflect the yellow portion are in the negative range or range around zero. This means that the red tone of the trout fillets depends on the amount of astaxanthin consumed.

During the experiment, for the performance parameters observed, no statistically secured differences are observed either between, or else within, treatments

(astaxanthin-containing powder, astaxanthin-containing extract in liquid form, synthetic astaxanthin, negative control).

It is found that astaxanthin-containing plants or parts of plants of the genus Tagetes or astaxanthin-containing extracts of astaxanthin-containing plants or parts of plants of the genus Tagetes are bioavailable in the pigmenting of trout as representatives of Salmonids and in addition do not lead to adverse effects on the biological performance of the trout.